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## REDACTORES

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## Determination of Inulin in Urine and Plasma.

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The basis of all recorded determinations of inulin in such concentrations as occur in clearance test is a hydrolysis of the material by acid reaction with subsequent determination of the hydrolysis products. These products, which are mainly fructose, are determined either by their reducing powers or colorimetrically with certain colour reactions which are more or less specific for fructose.

The colorimetric reactions described are a good deal more sensitive than determination by means of the reducing powers of the hydrolysis products; this fact of course suggests the use of the colorimetric methods when the concentration in the fluids to be analysed is low or the available quantity of fluid is limited.

Another circumstance which equally reduces the usefulness of methods based upon a reduction determination is that one often finds rather considerable blank reductions in certain biological materials (especially plasma), even after removal of glucose by fermentation, other substances with a not inconsiderable power of reduction being formed by the hydrolysis (*e. g.* by glycogenolysis?). For example, VAN SLYKE, HILLER and MILLER (1935) in dog plasma (precipitated with  $CdSO_4/NaOH$ ) found reductions equivalent to 3—5 mg % inulin. Other authors have found 3—25 mg % in man. If these values are correct, the blank reduction will form a large part of the total reduction, unless very high inulin concentrations are used — which is undesirable, for one reason on account of the expense. Consequently, variations in the value of

the blank reduction within the experimental period or errors in its determination may involve considerable error in the plasma-inulin determinations, even at concentrations of 40—60 mg %.

For these reasons, in the search for a suitable method I have ignored procedures based upon reduction determinations and have tested two of the colorimetric methods described in the literature.

A brief description of the colorimetric methods employed for inulin analyses may be given. They are based upon one of the following three foundations:

1. Diphenylamine reaction: Heating of fructose with diphenylamine in a solution containing hydrochloric acid leads to the formation of a blue colour.

The most widely used methods employing this principle are described by CORCORAN and PAGE (1939) and ALVING, RUBIN and MILLER (1939). A feature common to the procedure of these two groups of authors, in contrast to certain earlier methods using the diphenylamine reaction is the following: Hydrolysis and colour development proceed simultaneously. Ethylalcohol is added before hydrolysis and colour development, whereby the colour compound formed is held in suspension from the very moment it is formed. Apart from differences in the protein precipitation, the methods differ in the main only in the fact that the former authors apply heat from a boiling water-bath to open tubes for only 15 minutes, whereas the latter apply it for 60 minutes and to closed tubes. The methods differ also in respect of the technique employed for fermenting the glucose (glucose gives the same sort of colouration as fructose, though much less intense). The last-named authors remove the interfering glucose by fermentation in the plasma itself, a yeast-cell suspension being added; the resulting plasma dilution is corrected by means of a haematocrit determination on the yeast-cell suspension. CORCORAN and PAGE on the other hand ferment the glucose in the filtrate from the protein precipitate (SOMOGYI's  $ZnSO_4/NaOH$ ). SPÜHLER (1943) and JENSEN (1942) retain ALVING, RUBIN and MILLER's method almost unchanged, though JENSEN prefers propylalcohol to ethylalcohol.

2. Seliwanoff's reaction: Heating of fructose with resorcin in a solution containing hydrochloric acid leads to the formation of a red colour.

A modification of this method has been employed by ROE (1934) for determining fructose, and STEINITZ (1938) utilized this modification for inulin determination. Another modification of SELIWANOFF's reaction for inulin determination is described by HATZ and SZECSENYINAGI (1940). In both modifications hydrolysis and colour development proceed simultaneously, but in STEINITZ's method this takes place by heating to 80° C. for 8 minutes, whereas the other authors use 10 minutes, heating in a boiling water-bath and — in contrast to STEINITZ — only then adding alcohol.

3. Vanillin reaction: Heating of fructose with vanillin in a solution containing hydrochloric and phosphoric acid gives a red colour. HARLAY (1942).

In the search for a suitable method the following plan of work was observed: First of all, by employing pure aqueous inulin solutions an endeavour was made to find the most exact colorimetric method. Having found a satisfactory method, this was employed for the elucidation of certain other problems, viz.:

1. Recovery percentage of inulin in the filtrate after various precipitations.
2. Recovery percentage of inulin after fermentation.
3. The blank value in plasma and urine.

Colorimetry was performed with a Weka photoelectric colorimeter (a two-cell apparatus on the substitution principle described by HAVEMANN (1940)) using an incandescent bulb and colour filter.

### Colour Development on Aqueous Inulin Solutions.

Various modifications of the diphenylamine and Seliwanoff reactions have been tested.

a) *Diphenylamine reaction:* Both CORCORAN and PAGE and ALVING, RUBIN and MILLER employ the following procedure: To a solution containing 80 ml. conc. HCl and 110 ml. abs. ethyl-alcohol, 10 ml. of a 10 % solution of diphenylamine in abs. ethyl-alcohol is added immediately before use. Mix two parts of this with one part of inulin solution. As it was found, however, that the blank tests gave an increasing seagreen tint when one used an HCl-ethylalcohol which had been mixed for some time, or an old diphenylamine solution, the reagent was prepared fresh every day from its three constituents.

As the colour compound formed on heating 4—10 mg % inulin

solutions with this reagent displayed a tendency to precipitate as minute particles, the alcohol concentration was raised to 65 % of a 96 % ethylalcohol with corresponding reduction of the hydrochloric acid content. However, even with a reagent of this composition (500 mg. diphenylamine dissolved in 65 ml. 96 % ethylalcohol, with 35 ml. conc. HCl then added) a slight precipitation was observed now and then.

In advance CORCORAN and PAGE's procedure, with the heating of the mixture in open tubes, was considered to be unsuitable on account of the possibility of differences in the evaporation of alcohol and HCl in the various tubes, and in fact tentative tests confirmed that supposition. In experiments with ALVING *et al*'s type of tube, with a screw lid and rubber liner, there was a slight turbidity in the blank samples, although the rubber liners were thoroughly cleansed in soda lye; it was therefore decided to heat the mixture in sealed and carefully cleansed test-tubes in all experiments. The tubes were heated in a boiling water-bath.

Plotting the absorptions (measured with red filter RG<sub>1</sub>) observed, when an inulin solution is heated with twice the quantity of the diphenylamine reagent in a boiling water-bath, as functions of the boiling time, it was found that the colour absorption increases quickly at first; then more and more slowly; but even after 90 minutes' "boiling" the colour development has not reached its end-point. Longer heating was not thought practicable, and therefore it was decided to stick to the 60 minutes recommended by ALVING *et al*. However, the circumstance that colour development is still in progress has the effect that even slight temperature differences in the waterbath (*e. g.* owing to different barometric pressure on different days) cause the colouration to be somewhat different.

On carrying out decuplicate analysis with the technique described, all the samples being heated in the same water-bath, such deviations were observed, that the method, though it may serve for some purposes, cannot be considered ideal. The results of a decuplicate analysis made under these conditions may be given as an example: 4.89; 4.91; 4.96; 5.00; 5.01; 5.03; 5.04; 5.09; 5.16. Here the mean value is 5.006, the standard deviation 0.081 or 1.6 % of the mean value, and the standard error 0.025. Not infrequently, however, even among analyses from the same water-bath there are greater deviations than the above, especially in a positive direction, so it is doubtful if the values show a normal

distribution. The cause of this is not quite known; but as the diphenylamine reagent gives deep blue to green tints with a number of oxidizing agents (chromates, potassium permanganate, nitrates, etc.), the most scrupulous cleanliness with all glassware is necessary. For analyses heated in different water-baths and especially at different times there is usually greater deviation. Experiments with the use of propylaleohol instead of ethylaleohol made no improvement.

b) Seliwanoff's reaction: When testing this reaction the procedure described by ROE (1934) was followed. Various proportions of the ingredients in the resorcin-ethylaleohol-hydrochlorid acid reagent were tried until the following was decided upon: 100 mg. resorein dissolved in 60 ml 96 % ethylaleohol, whereafter 40 ml. conc. hydrochloric acid was added. One part of inulin solution (2.5 ml.) + two parts (5 ml.) reagent were heated in sealed test-tubes.

A series of tests of the same concentration were heated in a water-bath at 100° respectively 80° C. for various periods and then, after quick cooling to room temperature, the absorptions were measured with green, blue-green and blue filters.

Generally speaking, there is first a successive development of a cherry-red colour, which gradually tones into a lemon-yellow tint, which remains constant if the heating is continued. At 100° this colour development proceeds at a much greater velocity than at 80°, and the process is quickest at high concentrations of hydrochloric acid. In correspondence with this colour development it is observed that the colour absorption, measured with the aforesaid filters, first rises to a maximum and then gradually decreases to a constant minimum. The figure 1 illustrates these changes when the mixture is heated in a boiling water-bath and colorimetry undertaken immediately after cooling. Under such conditions the maximum absorption occurs after about 10 minutes; at 80° only after 15—20 minutes.

With the methods described in the literature, colorimetry is undertaken at times when there is still a red tint, but a test showed that this red tint, appearing for instance after 10 minutes at 100° is not constant, as the solution after cooling shows a gradual increase in the colour absorption. Even when colorimetry is undertaken immediately after cooling, the values obtained from a duplicate analysis with 10 minutes' boiling show greater deviation than in the above example of the diphenylamine reaction.

The various methods in the literature, with colorimetry on red, having failed to give satisfactory results, the possibility of applying colorimetry at the subsequent minimum, corresponding to the lemon-yellow tint was examined. To save time the tests were made only after heating in a boiling water-bath, because at lower temperatures the process takes longer to reach the end-point. In all the following readings a blue filter was used with a maximum transmission at about 4,500 Å and a cuvette with a layer thickness of 5 mm. The figure 1 shows, that at 100° the minimum is reached after about 45 minutes, but a period of 60 minutes was decided upon in order to be on the safe side.

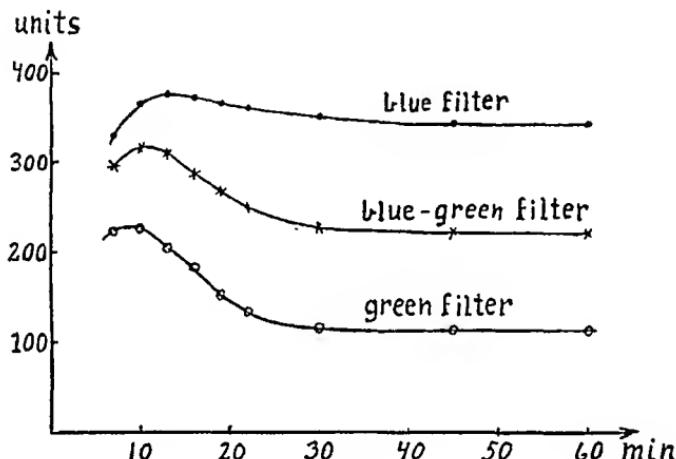


Fig. 1.

The following results show the accuracy obtainable by this procedure: 20 values obtained, 10 on each of two days, the analysis being made on the same inulin solution: First day: 4.94; 4.97; 4.97; 4.98; 4.99; 4.99; 5.00; 5.00; 5.02; 5.06, with the mean value at 4.992. Second day: 4.96; 4.98; 4.98; 4.99; 5.00; 5.00; 5.01; 5.02; 5.06; 5.08, with the mean value at 5.008. The standard deviation for the two series together is 0.035, or 0.7 % of the mean value; the standard error is 0.008. This shows that the method permits of determining inulin in aqueous solutions with satisfactory accuracy. As colour development has reached its end-point after 60 minutes' heating, the heating time is not critical as in the diphenylamine method, nor has it any effect on the results that the temperature of the water-bath is not exactly 100° C.

Like the diphenylamine reagent, the resorcin reagent is extre-

mely sensitive to oxidizing agents, for which reason the utmost cleanliness with all glass is necessary in this case also. It is also sensitive to ultraviolet rays, and therefore it must on no account be exposed to bright sunlight.

It was observed earlier (CORCORAN and PAGE; ALVING, RUBIN and MILLER) that glucose gives the same sort of colour development, though less intense than fructose (inulin) with diphenylamine reagents. Much the same occurs in the resorein reaction described above. The following table (1) shows that glucose gives a colour development corresponding to 7.9 % of the colour developed by the same amount of inulin. This of course means that when employing the resorein method with biological fluids containing glucose we must first remove the glucose or correct for the colour development it entails.

Table 1.

Glucose concentration mg %	Equivalent to an inulin conc. mg %	Thus 1 mg. inulin is equivalent to mg. glucose
8.125	0.66	12.3
16.25	1.27	12.8
24.375	1.92	12.7
32.5	2.58	12.6
65.0	5.11	12.7

Conclusion: On an average 1 mg. inulin is equivalent to 12.65 mg. glucose.

#### Recovery-% of Inulin in the Filtrate from Various Protein Precipitations on Plasma and Aqueous Inulin Solutions, and the Recovery-% of Inulin after Fermentation.

Regarding the suitability of various methods of protein precipitation the literature contains a number of contradictory opinions. The methods employed in inulin determinations have especially been  $Zn(OH)_2$ -precipitation *a. m.* SOMOGYI (1930, procedure 2) or  $Cd(OH)_2$ -precipitation *a. m.* FUJITA and IWATAKE (1931). Opinions are divided on the subject of the recovery of inulin by these precipitations. For example, ALVING, RUBIN and MILLER (1939) consider that both methods give "identical and correct values". STEINITZ (1938) finds that the  $Cd(OH)_2$ -precipitation results in a lower recovery than the  $Zn(OH)_2$  method, and apparently he considers that the latter gives a quantitative recovery in the filtrate. Similar views were advanced by RAPPAPORT (1937).

For the protein precipitation the present author employed

SOMOGYI's (1930, Procedure 2)  $Zn(OH)_2$  method, although having regard to the yeast blank value (v. i.) the reagents were adjusted to each other a little differently (50 ml.  $ZnSO_4/H_2SO_4$  reagent was titrated with phenolphthalein as indicator with 6.4—6.5 ml.  $NaOH$ , about 0.79 n).

For precipitation pipette 8 ml.  $ZnSO_4/H_2SO_4$  reagent, add to this 1 ml. analysis fluid (plasma, urine etc.) and finally 1 ml.  $NaOH$ ; mix thoroughly, centrifuge.

For fermentation I preferred the procedure of CORCORAN and PAGE (1939), with fermentation in the filtrate from the Somogyi precipitation, to fermentation in the plasma itself. The reason was that with the small quantities of plasma usually available (1—2 ml.) we must expect a not inconsiderable dilution error by direct fermentation on plasma by ALVING, RUBIN and MILLER's method.

In preliminary tests on solutions of glucose in the filtrate from Somogyi precipitations it was found that 2 ml. of a 10% washed yeast suspension (Baker's yeast from The Danish Distilleries) sufficed to remove at least 150 mg % glucose from 7—8 ml. Somogyi filtrate in 30 minutes at 30—35° C. Fermentation was therefore carried out in the following manner: 2 ml. 10% freshly washed yeast suspension was pipetted into centrifuge tubes and centrifuged for 20 minutes at 3,000 r. p. m., whereafter the supernatant fluid was removed completely by suction; on to the remaining yeast was poured the supernatant fluid from the Somogyi precipitation, and the yeast stirred until it formed a homogeneous suspension; after standing for 30 minutes at 30—35° the yeast was again centrifuged off and the clear supernatant fluid was used for the colour-reaction as described above.

For the recovery test we employed aqueous solutions and plasma with a known inulin content from which the glucose had been fermented.

The procedure was as follows: S being an aqueous solution of inulin of known concentration, the following solutions were prepared. 1) Water. 2) 1 ml. S + 10 ml. water. 3) 1 ml. water + 10 ml. fermented plasma. 4) 1 ml. S + 10 ml. fermented plasma. Six samples of each were precipitated as above *a. m.* Somogyi; three were taken from each group for fermenting in the manner described, whereas the other three were not fermented. In addition, 5) 1 ml. water + 9 ml. Somogyi filtrate, and 6) 1 ml. of the mixture 2 + 9 ml. Somogyi filtrate.

Table 2.

Sol. No.	1		2		3		4		5		6	
	un- ferm.	ferm.										
Equiv. to mg % inulin	0.0	0.7	52.6	50.8	0.2	0.5	53.3	51.4	0.2	0.2	53.6	

From Table 2, in which  $S = 600 \text{ mg \% inulin}$ , the recovery % is found to be:

- 1) Somogyi precip. on aqueous sol.  $\frac{(52.6 - 0.0) 100}{53.6 - 0.2} = 98.5 \%$ .
- 2) Somogyi precip. on plasma  $\frac{(53.3 - 0.2) 100}{53.6 - 0.2} = 99.4 \%$ .
- 3) Somogyi precip. on aqueous solution followed by fermentation  $\frac{(50.8 - 0.7) 100}{53.6 - 0.2} = 93.8 \%$ .
- 4) Somogyi precip. on plasma followed by fermentation  $\frac{(51.4 - 0.5) 100}{53.6 - 0.2} = 95.3 \%$ .

Other similar recovery experiments at other inulin concentrations have given analogous percentages; the following may be given as the average values for 1) 1.5—2% loss; 2) about 1 % loss; 3) about 6 % loss; 4) 4—5 % loss.

With  $\text{Cd(OH)}_2$  precipitation *a. m.* FUJITA and IWATAKE matters are very nearly the same as with Somogyi precipitation.

The loss of about 4 % caused by the fermentation process itself must be due to adsorption of inulin to the yeast. Dilution with intercellular water in the packed yeast cannot give errors of anything like this order. The circumstance that further purification of the inulin by repeated reprecipitations from alcoholic solutions makes no change in the size of this loss argues against the absorption of chromogen (non-inulin) substance in the yeast cells.

If when making inulin clearance determinations we carry out the fermentation on plasma as well as on the diluted urine as a matter of routine, the errors deriving from the fermentation will compensate one another in the calculation of the clearance; if it is a question of determining the absolute concentrations a correction for the fermentation loss is always necessary. Correction must always be made for the errors deriving from the precipitation of protein-containing and non-protein containing fluids.

### Blank Value in Plasma and Urine.

When employing  $Zn(OH)_2$  precipitation as described by Somogyi (50 ml.  $ZnSO_4/H_2SO_4$  reagent titrated with 6.7—6.8 ml. NaOH and then carrying out the fermentation as described above, it was found that Somogyi filtrates of both fermented plasma and water gave blank values of a not inconsiderable order. It was observed, however, that by using a somewhat stronger NaOH for the precipitation the blank value could be greatly reduced. This must be due to the yeast giving off less chromogenic material at the more alkaline reaction thus conferred on the Somogyi filtrates. Table 3 illustrates this.

Table 3.

No. of ml. NaOH used for titrating 50 ml. $ZnSO_4/H_2SO_4$	Blank value equivalent to mg % inulin when precipitating water	Blank value equivalent to mg % inulin when precipitating fermented plasma
6.45	0.2	0.5
6.55	1.2	0.5
6.90	3.9	0.5
7.10	7.8	1.0
7.30	8.2	6.0

It appears from Table 3 that in order to get the blank value pressed down to a minimum the NaOH solution used should be so strong that only 6.4—6.5 ml. of it is used for titration. Numerous analyses have shown that by this means one gets very low blank values both for plasma (from rabbit and man) and for urine (same objects) diluted to a degree suitable for clearance tests. As a general rule values of less than 1 mg % chromogen expressed as inulin are found for plasma, and as this value varies very little in the course of several hours, the blank value represents no perceptible source of error with plasma-inulin concentrations of about 50 mg %. As to the urines, if these are diluted to such a degree, that the inulin concentrations will be approximately the same as in plasma (for a healthy adult man to about 120 ml for each minute the urine is collected), one usually finds still lower blank values in a Somogyi filtrate.

### The Method in Practice.

Two elaborations of the method are given below. The first one, in which a fermentation is avoided, a correction for the chromogen effect of the glucose being inserted instead, is best for

glucose concentrations below 150 mg % and undoubtedly gives the more exact results when the glucose concentrations are low.

1) *Procedure without fermentation.*

Reagents: Resorein. Dissolve 100  $\pm$  1 mg. purest crystalline resorcin in 60 ml. 96 % ethylalcohol, then add 40 ml. conc. HCl pro analysi. Shake and cool. Prepare fresh for each series of analyses.<sup>1</sup>

Somogyi precipitation fluids:

a) Add 12.5 g. ZnSO<sub>4</sub> pro analysi to 31.25 ml. n/1 H<sub>2</sub>SO<sub>4</sub> per analysis and make up to 1,000 ml. with distilled water.

b) About 0.79 n NaOH.

When titrating with phenolphthalein as indicator 50 ml. of a) should take 6.4—6.5 ml. of b). (Of course, in this procedure one may also use the original strength of NaOH proposed by Somogyi.)

Centrifuge tubes prepared with heparin: To every 10 ml. centrifuge tube add 0.1 ml. 500 mg % heparin (Leo) = 125 Howell units; dry — turning occasionally — in a desiccator at 50—80° (or over a radiator). This quantity of heparin will definitely prevent the coagulation of 10 ml. blood for five hours at room temperature.

Reagents (except precipitation reagents) for blood-sugar tests  
a. m. HAGEDORN and NORMAN JENSEN (1923).

*Procedure:* Into a 10 ml. conical centrifuge tube pipette 8.00 ml. precipitation fluid a). With a pipette calibrated to contain 1.00 ml. add plasma (or diluted urine), sucking up and blowing out repeatedly. Finally, add 1.00 ml. precipitation reagent b).

Mix the contents thoroughly by turning on a clean finger; stopper and allow it to stand at least 10 minutes — or eventually over night.

Centrifuge twice five minutes, the interval between the two processes being employed for shaking gently in order to wash down the sediments adhering to the surface and the upper walls of the tubes.

Of the clear supernatant fluid pipette samples for determining glucose and total chromogen:

1) For glucose determination pipette a suitable quantity of fluid (usually 1 ml.); add this to 10 ml. distilled water in a Hagedorn boiling tube; then add 2 ml. of Hagedorn's K<sub>3</sub>FeCN<sub>6</sub> reagent, whereafter the glucose analysis proceeds in the usual manner.

<sup>1</sup> As cork and rubber contain chromogens this reagent should be prepared in a glass-stoppered bottle. The three ingredients should be kept in the same way.

If 1 ml. of the fluid was employed the ordinary Hagedorn-Jensen table shows directly the content of glucose in plasma (or diluted urine) in mg %.

2) For determining the total chromogen (inulin + glucose) the procedure is as follows: Of the supernatant fluid pipette 2.50 ml. into a perfectly clean test tube 15 × 150 mm. (cleansed by boiling in about 5 % hydrochloric acid-alcohol, followed by thorough rinsing in distilled water; the hydrochloric acid-alcohol can be used several times). Then with a Krogh's syringe-pipette add 5.00 ml. resorcin reagent. In doing so one should carefully avoid to place any reagent so high up in the tube that it becomes heated during the following sealing process. The tube is then sealed in a blow-flame. The tubes are heated for 60 minutes in a boiling water-bath, then cooled off to room temperature in running water. Then open the tubes (with a glass-file first, then break off). Before being opened they may stand at least an hour in a dark place without changing colour.

Colorimetry is carried out as already described in a photoelectric colorimeter with a blue filter in cuvettes with a layer thickness of 5 mm. For zero setting a sample with 2.50 ml. distilled water + 5.00 ml. resorcin reagent boiled for 60 minutes is used. Then take another sample which has passed through the entire analysis procedure as described, but in which 1 ml. distilled water is used instead of plasma (urine); this gives the blank value of the precipitation reagents.

How much the colour developed corresponds to in mg % inulin is read from an adjustment curve. This is constructed on the basis of the colours developed by pure aqueous inulin solutions when 2.50 ml. are boiled for 60 minutes in 5.00 ml. resorcin reagent. (It is convenient to employ the following concentrations: 10; 7.5; 6.25; 5.0; 3.75; 2.5; 1.25; and 0.612 mg %.) The concentrations are plotted as the ordinate, the number of units on the revolving drum of the colorimeter as the abscissa. As the mixture is diluted 10 times during precipitation, the chromogen concentration in plasma (or diluted urine) is ten times as high as that read from the curve.

The inulin concentration is calculated as follows:

a) in plasma:

$\text{mg \% inulin} = \text{measured chromogen concentration (in mg \%)} \cdot 0.079.$

$$\text{inulin} \cdot \frac{100}{99.2} - \text{glucose concentration in mg \%} \cdot 0.079.$$

b) in diluted urine:

$$\text{mg \% inulin} = \text{measured chromogen concentration (in mg \% inulin)} \cdot \frac{100}{98.3} - \text{glucose concentration in mg \%} \cdot 0.079.$$

The fact that the above procedure gives reliable results will be seen from an example such as the following: To 10 ml. fermented plasma 1 ml. of a solution containing 660 mg % inulin and 1,100 mg % glucose was added. By means of the above analysis procedure and calculation the following inulin concentrations have been found in the resulting mixture: 59.8; 60.0; 60.2; 60.4; 60.5 and 60.8.

2) *Procedure with fermentation.* This can be used with plasma-glucose concentrations up to 2,000 mg %.

*Reagents.* These are the same as those employed in Procedure 1.

Further a 10 % yeast-cell suspension: 50 g. fresh baker's yeast (Danish Distilleries) is washed three times with distilled water and then filled up to 500 ml with dist. water. Will keep fourteen days in a refrigerator.

*Preparing fermentation tubes.* These must be prepared fresh for every series of analyses. Of the above yeast suspension take a suitable quantity, which must again be washed three times before use. Pipette 2 ml. of the freshly washed suspension into 10 ml. conical centrifuge tubes with rounded bottoms. Centrifuge the tubes for 20 minutes at 3,000 r. p. m. *Immediately after* centrifuging draw off the supernatant fluid *completely* with a capillary tube in conjunction with a water suction pump.

*Procedure.* Plasma (or diluted urine) is precipitated in the same manner as in Procedure 1. Centrifuge for 10 minutes at 3,000 r. p. m.

The whole of the clear liquor above the sediment is poured into one of the fermentation tubes which have just been prepared. (If insignificant quantities of sediment are carried over it is of no consequence.)

With a small glass rod stir the yeast in the fluid until it forms a homogeneous suspension, then stopper the tube and allow it to stand — with occasional gentle shaking — for 30 minutes in a waterbath at 30—35°. Then centrifuge the stoppered tube for 10 minutes at 3,000 r. p. m.

Of the supernatant clear fluid now pipette 2.50 ml. into a clean test-tube, and continue as described above for determining the total chromogen.

The blank values for fermented plasma or diluted urine are determined in the following manner: 1) for Plasma: Plasma taken prior to the inulin injection is treated according to the method described above. 2) Diluted urine from a pre-period is treated in the same manner. (Carry out the dilution so that urine collected over a period just as long as the experimental periods is diluted up to the same volume as the urine from those periods.) The zero adjustment is the same as in Procedure 1.

The calculation of the inulin concentration proceeds as follows:

a) in plasma:

$$\text{Inulin concentration} = (\text{measured inulin conc. in mg \%}) - \frac{\text{plasma blank value in mg \% inulin}}{95.5} \cdot \frac{100}{95.5}$$

b) in diluted urine:

$$\text{Inulin concentration} = (\text{measured inulin conc. in mg \%}) - \frac{\text{blank value in diluted urine in mg \% inulin}}{94} \cdot \frac{100}{94}$$

This procedure also gives fairly accurate results; the following may serve as a representative example: To 20 ml. fermented rabbit plasma 2 ml. of an aqueous solution with a content of 468 mg % inulin and 5 % glucose were added. With Procedure 2 the following values for the inulin content were found: 41.5; 41.7; 41.9; 42.0; 42.1; 42.1; 42.2; 42.4; 42.5; 42.6. This gives a mean value of 42.1 against a real value of 42.5, or a difference of about 1 %. The values group about the mean value with a standard deviation of 0.37 or about 0.9 % of the mean value.

### Summary.

A colorimetric method for the determination of inulin in plasma and urine is presented.

It rests on the measurement of the yellow colour (absorption measurement at 4,500 Å) developed by heating inulin at 100° for 60 min. with resorcin in a solution containing ethyl alcohol and hydrochloric acid. This marks a real end point in the colour development process; the resulting colour compound is very stable and, contrary to the blue diphenylamine colour, it shows no disposition to precipitate.

The recovery percentages of inulin after protein precipitation and fermentation procedures have been determined. Further the blank values of plasma and urine.

Two procedures — one with and another without fermentation — are elaborated.

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## Inulin as an Indicator for the Extra-cellular Space.

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Various substances, some present in the organism, others to be injected, have been proposed for the measurement of the extracellular volume in the whole organism as well as in individual tissues, the last named determinations often being carried out to calculate the electrolyte composition of the intracellular fluid.

The common basis on which the calculations of the extracellular volume rests is the assumption that, when equilibrium has been attained, these materials will be present in the interstitial fluid in just the same concentration as in plasma ultrafiltrate. Different investigations support this assumption (PETERS (1935) and MAURER (1938)).

An ideal indicator of the extracellular fluid space should possess the following properties:

- 1) When sufficient time has elapsed for complete distribution the material must be found everywhere in the extracellular fluid and exclusively there and the concentrations of it must be uniform throughout the interstitial fluid and throughout plasma water, the ratio between the concentrations in the two compartments being a known fixed figure.
- 2) It must not be eliminable from the extracellular space (by excretion, storage, or decomposition).

Further if it is a foreign substance to be injected, the quantity which must be administered to get concentrations which may be determined with sufficient accuracy must

3) not possess so high an osmotic pressure, that it causes a perceptible displacement of water from the intracellular to the extracellular space.

4) not be toxic.

When complete distribution has been attained for an indicator of this kind, the extracellular fluid volume may be calculated as follows:

(1) For a non-electrolyte:

$$\text{extracell. vol.} = \frac{\text{quantity injected (or present in the organism)}}{\text{quantity in 1 ml plasma water}} \\ (\text{in ml.})$$

(2) For an electrolyte ion:

$$\text{extracell. vol.} = \frac{\text{quantity of ion injected (or present in the organism)}^1}{\text{quantity of ion in 1 ml plasma water} \cdot F} \\ (\text{in ml.})$$

$$\text{where } F = \frac{\text{ion concentration in interstitial fluid}}{\text{ion concentration in plasma water}}$$

If a substance is not known to be exactly confined to the extracellular space the neutral term "available space" should be preferred.

If we use these formulae for calculations before the distribution is complete, the figure calculated will vary with the point of time for the determination. These values we may name "volumes of distribution".

Naturally, to calculate a volume of distribution at a moment when the distribution is not yet complete may be considered illogical; nevertheless it has proved useful to do so, as a curve in which volumes of distribution are plotted against time (curve of distribution) represents a simple method of graphic illustration of the rate of distribution, moreover such curves make a direct comparison between the rate of distribution of different solutes possible.

The volume of distribution for a non-electrolyte is defined as the volume which should act as solvent for the injected quantity

<sup>1</sup> The precise expression here would be  
 $\frac{\text{injected quantity} - \text{plasma volume} \cdot \text{conc. in plasma}}{\text{quantity in 1 ml plasma water} \cdot F} + \text{plasma water volume}$ ,  
 however, the above given formula is a good approximation.

if the concentration was to be the same as in plasma water throughout the volume. Actually, before the distribution is complete there must of course always be places in which the concentration is less and eventually also places in which it is higher than in the plasma water.

In practice, it has proved impossible to find an indicator satisfying all the above mentioned requirements; at least every proposed substance is excreted in the urine.

Certain authors have therefore adopted the procedure of determining the urinary excretion of the substance administered in a single dose, and then in the calculation according to formula (1) or (2), replacing the injected quantity with the quantity retained at the end of the experimental period. In such a procedure, however, on account of the continuous excretion, an equilibrium between the concentrations of the indicator substance in plasma and interstitial fluid is never attained. For this reason the plasma concentration found is not a correct measure of the distribution; for instance, on a falling plasma concentration when this has passed the average interstitial concentration, the extracellular space is estimated too high. While errors from this source are small for substances (as SCN<sup>-</sup>), which are excreted slowly, they may become considerable for substances (inulin, sucrose) which are excreted quickly.

Therefore, if one wants to determine the available spaces for substances of this last category, a procedure must be used in which an equilibrium between plasma and interstitial fluid can be established. Theoretically two methods should be possible:

1) After a large initial dose the substance might be administered by steady, continuous infusion to keep the plasma concentration constant until distribution was complete.

2) The organs of excretion (The kidneys) might be removed prior to administration.

In practice, for quickly excreted, slowly diffusing substances the first possibility will be found unsuitable: at the end of a period long enough for complete distribution, it is found that the quantity retained represents only a small fraction of the quantity administered and therefore can be determined only with insufficient accuracy. Another source of error in the determination of the quantity retained is due to the fairly large quantities of these substances which, owing to their high urinary concentrations are accumulated in the urinary tract.

Now, in the course of some experiments on rabbits, including determinations of inulin clearance by continuous infusions, the impression was received that the inulin available volume was significantly smaller than the values given in the literature for other substances.

For the further investigation of this question, only method 2 appeared to be suitable. Hence the distribution of inulin in nephrectomized animals has been investigated. As a basis for comparison it was decided to determine the distribution of sulphocyanate, mainly because this is the substance most frequently employed by earlier workers.

### Experimental Technique.

The animals used in the experiments were 13 amyntalnarcotized (60–70 cg/kg.) male rabbits of 2.5 to 3 kg. and an ether-morphia narcotized male dog of 12 kg; prior to the experiment, except in one special case, the animals had been in a state of inanition for 18 hours, but with unlimited access to water.

On the day of the experiment the narcotized animals were strapped on their backs on an electrically heated operating-table. The left jugular and the right carotis were exposed and furnished with cannulae. Both kidneys were then removed through an abdominal section, whereafter the abdominal wall was carefully sutured.

About 5–6 ml. arterial blood was drawn for determinations of the blank value for the inulin and thiocyanate analysis. Then a solution of inulin and NaSCN in physiological NaCl-solution, was injected intravenously in the course of about 15 seconds; as a rule about 300 mg. inulin and about 200 mg. NaSCN were injected in experiments on rabbits. The experimental period was reckoned from the middle of the injection time. At suitable intervals samples of 5–6 ml. arterial blood were then taken, up to a total of 8. The blood was stabilized with heparine. The samples were centrifuged immediately after being secured and the plasma was pipetted off and employed for determining the concentrations of inulin and SCN<sup>-</sup>.

In three of the experiments a test was also made of the effect of infusing a rather large volume (100–120 ml.) of a sulphate-substituted Ringer fluid of the following composition: Na<sub>2</sub>SO<sub>4</sub>, 10 H<sub>2</sub>O 35 g.; K<sub>2</sub>SO<sub>4</sub> 0.34 g.; CaSO<sub>4</sub>, 2 H<sub>2</sub>O 0.35 g.; NaHCO<sub>3</sub> 0.20 g. with water added up to 1 litre. In these cases the chloride concentration was determined before infusion and in the subsequent blood samples.

In two experiments it was possible after the termination of the experimental period to aspirate up to 4 ml. slightly blood-tinged peritoneal fluid, which was analyzed for inulin and SCN<sup>-</sup>. Twice the concentrations of these substances in tendon tissue determined, the Achilles tendons being excised during the experiment in these instances.

### Technique of Analysis.

*In blood plasma:* The method employed for determining *inulin* was that published recently by the author (KRÜHØFFER 1945 a Procedure 2).

The  $SCN^-$  concentration was determined by a slight modification of CRANDALL and ANDERSON's method (1934): Pipette 7 ml. distilled water into a 10 ml. centrifuge tube; with a pipette calibrated to contain add 1 ml. plasma under repeated suction and blowing, then add 2 ml. 30 % trichloracetic acid, mix thoroughly, leave for 10 minutes, centrifuge. Of the clear supernatant fluid pipette 3 ml., to which immediately before colorimetry add 4 ml. of CRANDALL's reagent (50 g ferrinitrate + 25 ml. conc.  $HNO_3$  + distilled water to 1 litre). Exactly 1½ minutes after adding the reagent take the colorimetric reading. (In the experiments here described colorimetry was carried out in a photoelectric absorptometer — Weka — described by HAVEMANN (1940), using a green filter BG<sub>7</sub>, and a cuvette layer thickness of 5 mm.) Read the result from an adjustment curve designed for known aqueous solutions (adjusted to silver nitrate), known concentrations being plotted along the ordinate and the corresponding readings along the abscissa. For plasma multiply by 10 on account of the dilution by deproteinization, and introduce a correction of —1.5 % for the concentration of the supernatant fluid due to the fact that the protein sediment has a certain volume.

The chloride in plasma was determined *a. m.* KEYS (1937).

For peritoneal fluid the procedure was centrifuging to remove the small content of erythrocytes and then analyzing exactly as for plasma.

The excised tendons were immediately freed of muscle, cut into small pieces and, after freezing in liquid air, pounded in a mortar. A suitable quantity was weighed off into a centrifuge tube and precipitated in the same manner as plasma. After standing for 18 hours in the refrigerator the sediment was centrifuged off and the supernatant fluid was analyzed for inulin and  $SCN^-$ .

*Determining the Ratio of the Diffusion Coefficients for  $SCN^-$  and Inulin.* The technique here was that described by NORTHRUP and ANSON (1929). The apparatus was a diffusion chamber with a volume of 24.7 ml. provided with a Jena glass filter disc No. G<sub>4</sub> with an active diameter of 30 mm. and a thickness of about 2 mm. The diffusion experiments were made at 37° C. The inulin employed was the same as that in the animal experiments, a triple alcohol-reprecipitated material from SCHERING-KAHLBAUM: "Inulin reinst". The inner fluid employed was an aqueous solution containing 1,500 mg % inulin and 700 mg % NaSCN.

Under these conditions the ratio between the diffusion coefficients  $\frac{D_{SCN^-}}{D_{inulin}}$  proved to average 5.60 ( $\pm 1\%$ ).

### Calculations.

The formula given previously (1) was employed for calculating the distribution volumes for inulin, the concentration in plasma water was calculated as plasma conc.  $\cdot \frac{100}{95}$ .

The calculation of the available volume for  $\text{SCN}^-$  was made according to formula (2), the same correction being made for the water content in plasma, whereas it was elected to put  $F = 1$ . This latter choice was made despite the fact that experiments by ROSENBAUM and LA-VIETES (1939) and the author's own tests of peritoneal fluid showed that  $F < 1$ . By taking  $F = 1$ , however, the chance of estimating the  $\text{SCN}^-$  available space too high was definitely avoided.

In the experiments where the chlorides of the organism were diluted by sulphate infusion, a volume containing chlorides capable of being diluted (named "volume of dilutable chlorides") was calculated according to the following formula:

$$\text{Cl-vol}_t \times \text{plasma-Cl}_0 = (\text{Cl-vol}_t + N) \times \text{plasma-Cl}_t,$$

where plasma- $\text{Cl}_0$  means the plasma-chloride concentration prior to the infusion; plasma- $\text{Cl}_t$  the same the time  $t$  after termination of the infusion; Cl vol. $_t$ , the volume of dilutable chlorides calculated from the dilution of plasma chloride existing at the time  $t$ , and  $N$  the infused number of ml. sulphate solution.

### Results.

Fig. 1 shows the result of an experiment in which a rabbit (No. 17) was intravenously injected with 196 mg. inulin and 193 mg. NaSCN in 10 ml. fluid. The falling curves represent the concentrations (in mg %) of inulin and NaSCN = calculated for an ultrafiltrate of plasma as functions of the time after the injection; the concentrations are read on the ordinate scale on the left. The ascending curves represent the volumes of distribution for inulin and  $\text{SCN}^-$  calculated in the manners described above; they are read from the ordinate scale on the right, where their values in per cent. of the body weight can also be found.

It is evident that the volumes of distribution for  $\text{SCN}^-$  throughout are larger than the simultaneous volumes of distribution for inulin. However, the two volumes at the same point of time are not directly comparable, as inulin diffuses at a much slower rate than  $\text{SCN}^-$ . The experiments referred to above showed that the coefficient of diffusion for  $\text{SCN}^-$  under the said conditions is 5.6 times higher than for inulin. If we presume (and it may be

## ultrafiltrate

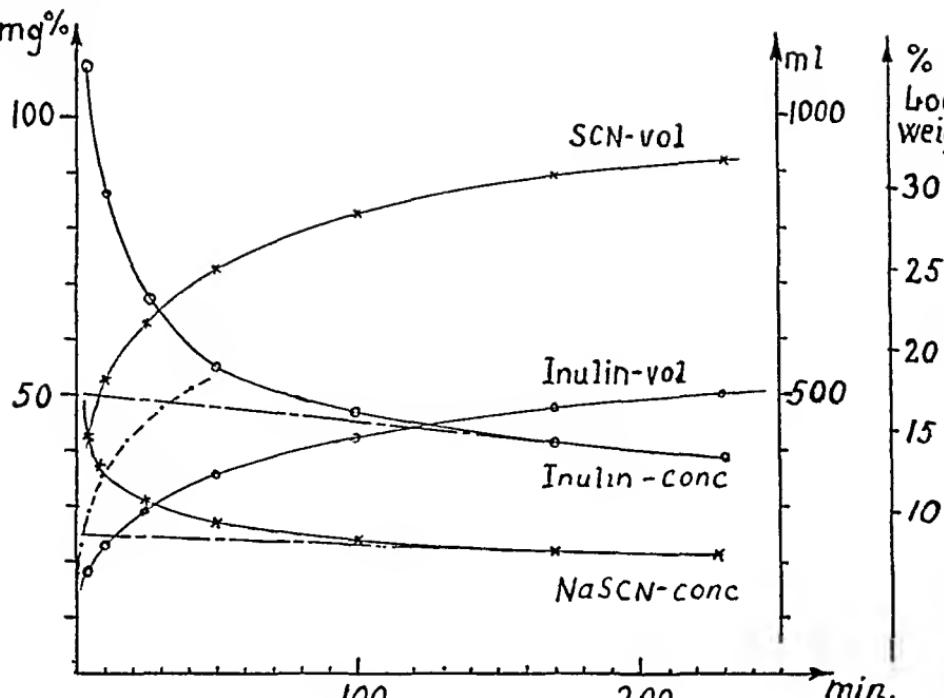


Fig. 1.

done with good approximation) that this value also applies to the organism and to the concentrations employed in the animal experiments, we can from the inulin distribution curve construct the distribution curve according to which in theory  $\text{SCN}^-$  would necessarily be distributed if finally it was distributed in the same volume as inulin and both substances were distributed in this volume by diffusion alone. The points on this theoretical distribution curve for  $\text{SCN}^-$  are found as follows: When for instance 100 minutes have elapsed the inulin has distributed itself over 420 ml., consequently at the time  $\frac{100}{5.6} = 18$  minutes  $\text{SCN}^-$  should have distributed itself in the same volume.

On fig. 1 will be found the constructed, theoretical distribution curve for  $\text{SCN}^-$  (—·—·—). If the substances are distributed not only by diffusion but also by convection (by movements of fluid in the interstitial space), the theoretical curve of distribution for  $\text{SCN}^-$  will lie nearer the inulin curve. Thus it appears very clearly that the thiocyanate ions distribute themselves in a much

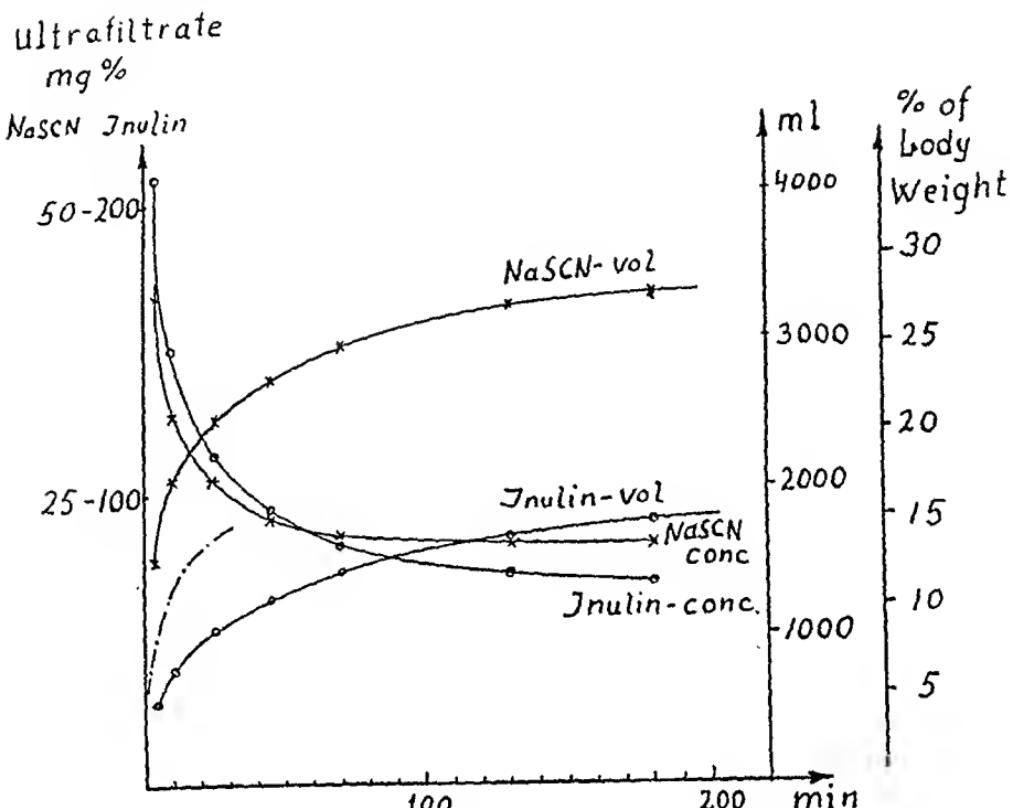


Fig. 2.

larger volume than the inulin, for example in the above experiment already after 41 minutes in a volume of at least 185 ml. larger than could be theoretically expected under the said conditions. (In part of the distribution volume — *i. e.* in plasma — the distribution does take place by convection; but as this proceeds very rapidly, it will scarcely affect the course of the curves at all after the first few minutes; *cf.* KRUHOFFER (1945 b).)

Corresponding results were found in other experiments with rabbits and in a single one with a dog. The results of the latter appear from fig. 2. The dog, which weighed 11.8 kg was injected intravenously with 1,200 mg. inulin and 685 mg. NaSCN in 20 ml. fluid in the course of 10 seconds.

The data included in table 1 will give an impression of experimental results obtained in all the experiments performed. This table shows the various distribution volumes as a percentage of the body weight at the time  $t$  minutes after the injection of inulin and NaSCN. The results at  $t = 30$  and  $t = 150$  minutes require

Table 1.  
*Distribution volumes in percentage of the body weight.*

Exp. No.	Animal's weight (kg)	Vol. of distrib. for Inulin			Vol. of distrib. for SCN-		
		t = 0	t = 30	t = 150	t = 0	t = 30	t = 150
<b>Rabbits</b>							
7	2.8	—	—	14	—	—	27.5
10	2.8	—	—	14	—	—	26.5
9	3.0	—	—	14.5	—	—	27
11	2.9	—	—	14.5	—	—	26
14	2.5	14.5	11.5	15.5	28	24.5	31
15	2.8	—	11	—	—	25	—
4	2.5	15	12	16	28.5	25	30
17	2.9	13.5	11	16	26.5	22.5	30
8	3.0	—	—	16.5	—	—	29
13	3.0	15.5	13	16.5	27	25.5	28
6	2.0	—	—	17	—	—	29
5	2.5	16.5	13	19	29.5	27	31.5
Mean values		15	11.9	15.8	27.9	24.9	28.7
Rabbit Dehydrat.	3.0	—	8.5	11.5	—	19.5	23
		—	—	9.5	14.5	—	22.5
Dog	—	—	—	—	—	—	27.5
16	—	—	—	—	—	—	—

no explanation. The results at  $t = 0$  were arrived at by the same procedure as that employed by KROGH (1938), *i. e.* by extrapolation back to the time 0. Strictly speaking, of course, this procedure is not permissible, as at no time do the curves for the concentration fall become rectilinear; but as after the first rapid fall they do assume a lesser curved course, one can by means of the extrapolation form an idea, if a rough one, of the concentration in which the substance would be found if the "rapid phase" of the distribution had taken place instantaneously. Of course, the volume calculated from this would not represent the final distribution volume, and at most it can only be of interest as a basis for comparison with other series of experiments. The table clearly illustrates how important it is to know the distribution time when it is required to judge and compare results obtained. It will also be seen that the dispersion in the results from rabbits, given the same pre-experimental treatment, is moderate.

In experiment 12 a dehydration was intentionally produced: the rabbit was first starved for 24 hours, whereafter the pylorus

was ligated and the animal was left in a cage for 30 hours with no opportunity of ingesting water or food or of coprophagy. After the contents of the stomach with 570 mg. chloride had been withdrawn through a tube, the experiment was performed with the usual technique. The values of the various volumes of distribution lie distinctly below the normal average.

Fig. 1 and 2 show that at the end of the experimental period the inulin and SCN<sup>-</sup> concentrations are still falling. The question then arising is whether this is because the distribution is not completed at this juncture or the substances are eliminated in the organism. In some cases the experiments were therefore extended over a much longer period. Here the practical difficulty was encountered that the nephrectomized rabbits could generally not be kept alive for more than 7—10 hours. In the experiment (No. 9) carried out over the longest period, 12 hours, it was found that the SCN<sup>-</sup> concentration had practically become quiescent; but at the end of the experiment there was still a slight fall in the inulin concentration. After 12 hours the inulin was found to be distributed in 18 per cent., the SCN<sup>-</sup> in 30.5 per cent. of the body weight.

As to an eventual elimination of inulin, attention was given especially to two possibilities: an extra-renal excretion and an absorption into the reticulo-endothelial system. HAYWOOD and HÖBER (1937) found that inulin could be excreted through the isolated, artificially perfused liver of a frog. Therefore in a few cases after the termination of the experimental period the gall-bladder bile was analyzed for inulin (after removing interfering pigments by means of carbon absorption in acetic acid solution). It was found that the content of chromogen substances (by the usual resorcin reaction) was only insignificant. In order to examine the possibility of absorption into the reticulo-endothelial system a couple of experiments were made in which the system was blocked by the injection of a dye. JACOBSEN and PLUM's method (1945) of injecting 10 ml. 1 % trypane blue intravenously three or four hours before commencing the actual experiment was employed. As will be seen from fig. 3, which illustrates the results of such an experiment (No. 13), this caused no demonstrable change in the course of the distribution curves. In other words, there was nothing to support the assumption that inulin is eliminated in the organism.

In fig. 3 is also shown the result of a sulphate infusion. In this

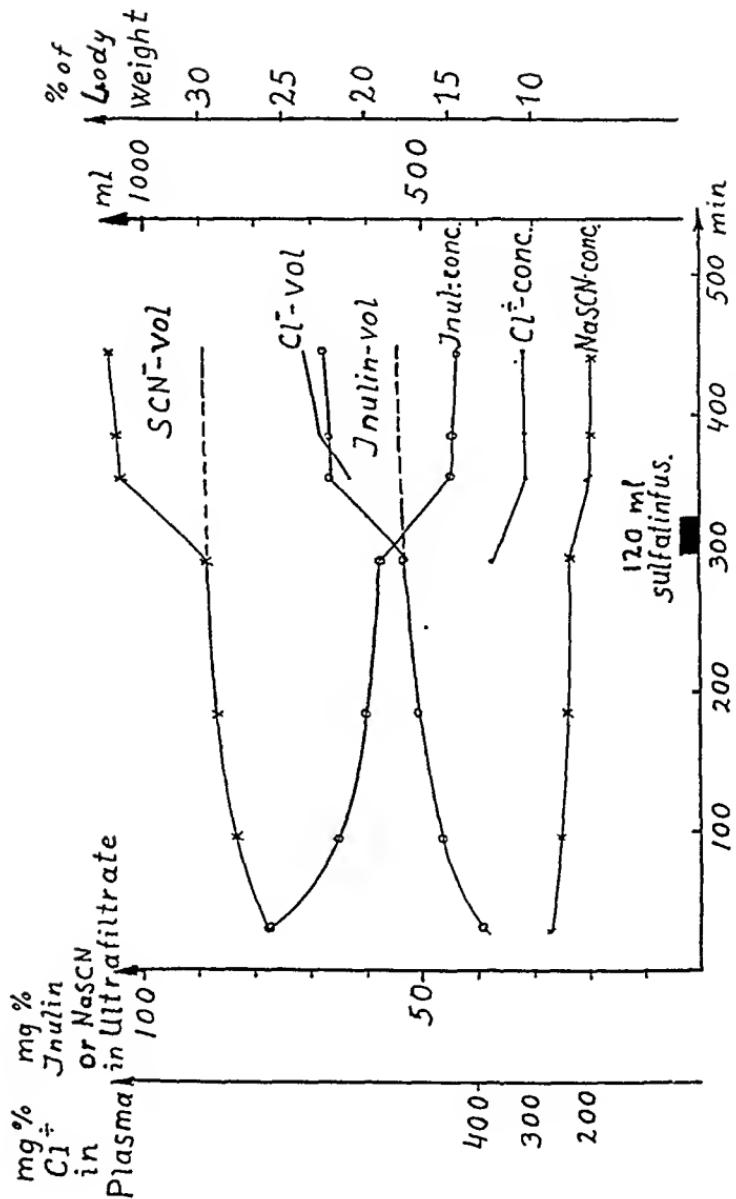


Fig. 3.

experiment, after most of the distribution had taken place (295 minutes), 120 ml. of the previously described sulphate solution was infused intravenously in the course of 25 minutes. As will be seen from the figure, this brought about an increase of the volumes of distribution for inulin and  $\text{SCN}^-$ . As regards the former the increase is only very slightly larger than the infused volume,

whereas the increase of the volume of distribution for SCN<sup>-</sup> is somewhat larger. The sulphate infusion moreover involves a dilution of the chloride in the organism, and, as already stated, this permits of a calculation of the volume of dilutable chlorides; the results of such calculations are also included in the figure. As the diffusion coefficients of chloride ions and thiocyanate ions are of the same order, these volumes are best comparable with the distribution volumes for SCN<sup>-</sup>, viz. by comparing a distribution volume for SCN<sup>-</sup> found after a certain period after the SCN<sup>-</sup> injection with a volume of dilutable chlorides after a corresponding period subsequent to the sulphate infusion. The volume of dilutable chlorides is then found to be a good deal smaller than the corresponding distribution volume for SCN<sup>-</sup> but significantly larger than any distribution volume for inulin.

In two similar experiments (Nos. 10 and 11) parallel results were obtained. In Experiment 10 the volume of dilutable chlorides were calculated 30, 90 and 170 minutes after terminating the sulphate infusion (120 ml.) they were found to be 20.9, 22.6 and 23.6 per cent. of the body weight, and in experiment 11: 30, 60 and 120 minutes after terminating the sulphate infusion (120 ml.) they were found to be 20.9, 22.6 and 23.6 per cent. of the body weight.

In two experiments the *peritoneal fluid* was analyzed at the end of the experiment immediately after the final blood sample was drawn. The results were as shown in table 2. In this table the concentrations theoretically to be expected in ultra-filtrates of plasma are calculated as follows: for inulin, by multiplying the plasma concentration by  $\frac{100}{95}$  and for SCN<sup>-</sup> (expressed as NaSCN) by multiplying by  $\frac{100 \cdot 100}{95 \cdot 95}$ .

From the table it will be seen that in both cases the inulin concentration in the peritoneal fluid lies a few % above the theoretically expectable concentration for an ultra-filtrate. The explanation of this is perhaps that the analyses were made at a time when the inulin concentration in plasma was still falling and balance not yet attained. As to the thiocyanate concentration it was found to lie about 10 % below what would be expected of a monovalent anion, which agrees very well with the results obtained by ROSENBAUM and LAVIETES (1939).

Table 2.

Distribution between plasma and peritoneal fluid.

Exp. No.	Minutes after start of exp.	Material	Plasma conc. in mg %	a = theoretic ultrafilt.	b = Cone. found in periton. fluid (mg %)	$\frac{b}{a} \cdot 100 =$ found in % of theoret.
4	515	Inulin $\text{SCN}^-$ (exp. as $\text{NaSCN}$ )	57.0	60.0	61.5	102.5 %
			25.0	27.7	25.0	88.5 %
5	430	Inulin $\text{SCN}^-$ (exp. as $\text{NaSCN}$ )	50.2	53.0	55.5	105 %
			21.3	24.0	21.9	91.5 %

As previously stated, in two cases analyses were made on tendon tissue excised in the course of the experiments. In experiment No. 14 (rabbit) the tendon was excised 150 minutes after commencing, when the inulin concentration in the plasma water was found to be = about 77 mg %; on analysis the tendon tissue was found to contain 17.8 mg % inulin, which corresponds to a content of about 23 ml. ultrafiltrate per 100 g. tendon. In the dog experiment (No. 16) the results were similar; the tendons were excised 130 minutes after commencing, at a time when the plasma concentration of inulin was 70.2 mg % corresponding to a concentration in an ultrafiltrate calculated in the above mentioned manner of about 73 mg %; at the same time 11 mg % was found in the tendon tissue. The corresponding figures for the  $\text{SCN}^-$  concentrations were about 21 mg % and 8.3 mg %, which means that in 100 g. tendon tissue there should be a content of 15 ml. ultrafiltrate according to the inulin distribution ratio or of 40 ml. according to the ratio of the thiocyanate ions. As the water content of the tendons is about 65 % most of which is extracellular owing to the histological structure, it will be seen that after the period mentioned the inulin was not nearly distributed throughout the extracellular space, whereas in the last experiment the thiocyanate ions had attained to a much higher distribution ratio. However, the tendon analyses embody not insignificant sources of error, for which reason the results must be judged with some reserve.

### Discussion.

Before judging the results arrived at in this work it may be of value to survey certain results obtained with other indicator substances by earlier workers.

A great part of the determinations of the "extracellular volume" has been based on the content of sodium and chloride, the postulate having been set forth that these substances were distributed evenly and exclusively in the extracellular space.

However, recent publications have made the correctness of this assumption very unlikely and in the following some of the more significant arguments, which have been raised against the theory that these two substances are true indicators for the extracellular volume, shall be summed up.

1) If the ratio m. eq. Na/m. eq. Cl in certain tissues is higher than the corresponding ratio in an ultrafiltrate of plasma, both sodium and chloride cannot be true indicators of the extracellular volume of those tissues and most probably sodium gives values that are too high. (There is a possibility, however, that chloride, contrary to sodium, may not permeate into extracellular structures (collagenous fibrillae, etc.).)

Moreover, if the ratio Na/Cl for another tissue of the same kind of animal (or the same tissue under other conditions) is found lower than in plasma ultrafiltrate it is most probable that both substances give too high values for the extracellular volume in the organism as a whole.

HARRISON, DARROW and YANNET (1936) investigating the electrolyte content of total bodies of rabbits, monkeys and dogs found the Na/Cl ratio on an average 25 % higher than in plasma ultrafiltrate. In bone and cartilage tissues the ratio Na/Cl has repeatedly been demonstrated highly to exceed that of plasma ultrafiltrate. (HARRISON (1937), IOB and SWANSON (1938), HARRISON, DARROW and YANNET (1936).)

On the other hand, for certain other tissues Na/Cl has been found lower than in a plasma ultrafiltrate: in connective tissue (MANERY, DANIELSEN and HASTINGS — 1938), in gastric and intestinal mucosa (MANERY and HASTINGS — 1939), in liver (YANNET and DARROW — 1940) and in immature rat uteri (TALBOT, LOWRY and ASTWOOD — 1940).

In striated muscle HASTINGS and EICHELBERGER (1937)

always found an excess of sodium over chloride, while MILLER and DARROW (1940) found Na/Cl capable of varying: at low potassium content of the muscles sodium was in excess of chloride whereas at high potassium contents the reverse was the case. (HEPPEL (1939) observed that muscle potassium could be replaced by sodium at low plasma potassium concentrations).

2) The permeability of certain cells which may easily be examined may serve as a guide.

It is well known that erythrocytes contain chloride (and small amounts of sodium), and that both the glandular cells of the intestinal tract and the tubular cells of the kidneys are permeable to these ions. Thus by analogy it seems probable that other cell membranes are also permeable to these ions.

3) If in one or other tissue the available space calculated for a certain substance is obviously in disagreement with that determined by histological investigation this substance cannot be used as an indicator for the extracellular space in that tissue. For example, when MANERY and HASTINGS (1939) found that calculated on the chloride content and expressed as a percentage of the *blood-free* and *fat-free* tissue the extracellular phase amounts to 22 % in liver, 32 % in spleen and 50 % in kidney (rabbits) these values seem incompatible with the histological structure of these tissues, even if for the kidney one makes a suitable correction for chloride contained in the tubular urine. The same applies to sodium. Analogous figures for cats' organs will be found in a publication by AMBERSON, NASH, MULDER and BINNS (1938).

4) As a true indicator for the extracellular volume must be found in that volume exclusively and moreover only in a free state, the concentration of such a substance in the tissues should vary proportionally with the concentration in plasma (when sufficient time for complete distribution is allowed for). If in certain tissues a smaller or larger fraction of the indicator does not readily follow the variations in the plasma concentration, that indicator will beyond doubt "indicate" a volume larger than the extracellular volume in these tissues.

Here the fine plasmapheresis experiments of the last-named four authors are particularly illustrative. By infusing sulphate solution i. v. while simultaneously depleting for blood a gradual fall in plasma chloride to very low values was produced. Whereas the chloride in some tissues (heart, liver, kidneys) fell proportionately with plasma chloride, other tissues (stomach,

spleen, pancreas, tendons, skin and especially the central nervous system) were found to contain a smaller or larger fractions of chloride which was not removed.

YANNET and DARROW (1940) found that muscles and liver also contained small amounts of not-readily diffusible chloride.

(As pointed out by MANERY, DANIELSEN and HASTINGS (1938) such nonfreely diffusible electrolytes need not be localized intracellularly, they may also be bound to extracellular structures (in larger amounts than their water contents can account for).)

Thus it seems reasonable to conclude that the available spaces calculated from sodium or chloride *naturally contained* in the organism and individual tissues are larger than the actual extracellular volume. Therefore, it is also to be supposed that the volumes determined by *administration* of these substances (eventually as isotopes), are too large.

Logically, if the available spaces calculated from experiments with other indicators turn out to be larger or equal to those calculated on the basis of sodium or chloride they must be considered larger than the true extracellular volume.

Apart from  $\text{Na}^+$  and  $\text{Cl}^-$ , the substances which have been proposed for the determination of the extracellular volume are chiefly  $\text{SCN}^-$  and  $\text{Br}^-$ ; determinations have also been published with  $\text{SO}_4^{--}$ , Sucrose and  $\text{Mg}^{++}$ .

Regarding bromide, as WEIR and HASTINGS have shown that it is distributed in exactly the same manner as chloride in all tissues (except the central nervous system) we should expect an available space identical with that of chloride.

Among the others, judging from the permeability found in erythrocytes, glandular cells of the intestinal tract and the tubular cells of the kidneys,  $\text{SO}_4^{--}$  should be expected to be a rather good indicator while sucrose (like inulin) should be ideal.

In table 3 a number of results from the literature are summarized. It contains both available spaces calculated from the content of  $\text{Na}^+$  and  $\text{Cl}^-$  in the organism and volumes of distribution calculated from substances *administered* (usually intravenously and always in a single dose).

In the case of administered substances the results are not directly comparable as the distribution volume depends on the time elapsed after the injection and on the diffusion rate of the substance in question. Further the results recorded were arrived at by different and not equally correct formulae and finally it

Table 3.

*Determinations of the volumes of distribution for different materials = N calculated 1) from the content or 2) from a single dose after a distribution time — t. (cited from the literature). Results in % of body weight.*

N	Man (healthy)	Dog	Rabbit	Cat
Cl <sup>-</sup> from content	—	26—28 % <sup>1</sup>	25, 28 and 28 % <sup>1</sup>	ca. 23 % <sup>2</sup> ca. 32 % <sup>3</sup>
Na <sup>+</sup> from content	—	37 % <sup>1</sup>	32—37 % <sup>1</sup>	—
<sup>24</sup> Na from dose	—	—	28 % <sup>3</sup> t = 3 min. 29 % <sup>4</sup> t = not stated	—
SCN <sup>-</sup> from dose	20—28 % <sup>5</sup> t = 60 min. or more ca. 19 % <sup>6</sup> t = ca. 30 min. <sup>6</sup>	ca. 30 % <sup>7</sup> t = 60 min.	ca. 26 % <sup>8</sup> t = 0 min. <sup>9</sup>	ca. 29 % <sup>6</sup> t = 30 min.
Br <sup>-</sup> from dose	23—31 % <sup>10</sup> t = 1½—24 hours	24—38 % <sup>10</sup> t = 1—30 hours 21—30 % <sup>11</sup> t = 6—497 min.	—	—
SO <sub>4</sub> <sup>2-</sup> from dose	20—28 % <sup>5</sup> t = 60 min. or more	21—32 % <sup>12</sup> t = 42—79 min.	—	—
Sucrose from dose	17—20 % <sup>5</sup> t = 90—120 min. 1) 17.7—20.7 % <sup>13</sup> t = 1—6 hours 2) 19.7—26.4 % <sup>13</sup> t = 1—6 hours	—	—	—
Mg <sup>++</sup> from dose	—	19—26 % <sup>12</sup> t = 42—79 min.	—	—

<sup>1</sup> HARRISON, DARROW and YANNET (1936). <sup>2</sup> AMBESON, NASH, MULDER and BINNS (1938), calculated from results in table 1 and protocol in the following way:  
 $2,400 \cdot 106/100$

$= 565$  m. <sup>3</sup> HAHN, HEVESY and RABBE (1939). <sup>4</sup> MANERY  
 $4.1 \cdot 100/95 \cdot 100/95$  = 565 m. <sup>5</sup> LAVIETES, BOURDILLON and KLINGHOFFER (1936). <sup>6</sup> STEWART  
 and ROURKE (1941). <sup>7</sup> SUNDERMANN and DOHAN (1941). <sup>8</sup> LANDS, CUTTING and  
 LARSON (1940). <sup>9</sup> KROCH, A. (1938), the plasma concentration at  $t = 0$  was deter-  
 mined by extrapolation. <sup>10</sup> BRODIE, BRAND and LESKIN (1939). <sup>11</sup> WINKLER  
 and SMITH (1938). <sup>12</sup> SMITH, WINKLER and SCHWARTZ (1939). <sup>13</sup> KEITH and  
 POWER (1937).

must be remembered that determinations on falling plasma concentrations are apt to give too high values.

Generally speaking, it is however seen from the table that there are scarcely any of the other substances that show a volume of distribution definitely lower than chloride, though sulphate and sucrose have a slight tendency in that direction, thus it should be supposed that all are distributed beyond the extracellular space.

The present work shows that in the rabbit and dog inulin becomes distributed in a perceptibly smaller volume than SCN<sup>-</sup> (and Cl<sup>-</sup>), and it is a well-known fact that inulin does not permeate the erythrocytes and cannot be assumed to pass into the tubulus cells of the kidneys. This work has also shown that it does not permeate liver cells (rabbit).

The question now is whether the available volume for inulin is a true measure of the extracellular space?

The answer cannot be given definitely in the affirmative, but it is justifiable to suppose that it is.

On the one hand, the available volume for inulin is scarcely too large to represent the extracellular space; it is distinctly smaller than the other "volumes" which several authors consider should represent the extracellular space. Moreover, inulin's higher molecular weight makes it very probable *a priori* that it does not permeate the cells of the organism.

On the other hand, it appears from the present work that inulin is capable of distributing itself regularly between plasma water and interstitial fluid — see the analyses of peritoneal fluid. Therefore it would be unnatural to suppose that the inulin does not distribute itself throughout the whole of that part of the extracellular fluid which is found extrastructurally.

However, there is a possibility that the inulin does not make its way into the intrastructural part of the extracellular space, by which is meant the fluid in structures such as collagenous fibrillae etc.

The above mentioned investigations on the volume of distribution of inulin in tendons might be expected to elucidate this question.

As it will be remembered, in two experiments it was found that after 150 min. respectively 130 min., inulin was distributed in 23 respectively 15 ml of the 65 ml total water contents in 100 g tendon tissue. Thus in both cases, in the time allowed for distri-

bution, inulin had by no means spread into the whole of the extracellular space in tendons; on the other hand, at any rate in the former case, inulin was distributed in a larger volume than the extrastructural water in tendon tissue can be imagined to constitute.

Consequently it seems justifiably to assume that the inulin, if only it is given sufficient time, will distribute itself over the whole extracellular space of the tendons. A long time will be required, however, owing to the sparse vascularization of tendon tissue.

Even if inulin must be assumed to be a correct indicator-substance for the extracellular space, its low diffusion rate will be a very considerable handicap to its practical application for determining the extracellular space in total organism as well as in individual tissues (especially in those with sparse vascularization). In the case of non-nephrectomized animals it can only be employed for determining the extracellular space in individual tissues after protracted, continuous inulin infusions.

As will have been seen, the thiocyanate ions distribute themselves in a volume far exceeding what can theoretically be calculated from the distribution curve of inulin. Provided that inulin distributes itself only in the extracellular space and throughout the whole of it, it will therefore be necessary to assume that  $\text{SCN}^-$  also distributes itself into a part of the intracellular fluid of the organism. For instance, in the experiment illustrated by fig. 1, after only 41 minutes  $\text{SCN}^-$  was found to be distributed in a volume 185 ml. larger than could theoretically be expected. If the erythrocyte volume of this rabbit is put at 80 ml., the water phase in these 80 ml. will be about 50 ml. Thus in addition to a (well known) distribution in the erythrocyte fluid there must also be a penetration by  $\text{SCN}^-$  into other cells of the organism.

### Summary.

A number of results are quoted from the literature, showing that the available volumes for  $\text{Cl}^-$  and  $\text{Na}^+$  are larger than the extracellular volume; the author gives a tabulated summary of literature references to the size of the available volume for these substances and the volumes of distribution for these and  $\text{SCN}^-$ ,  $\text{Br}^-$ ,  $\text{SO}_4^{2-}$ , sucrose and  $\text{Mg}^{++}$ .

In 13 nephrectomized rabbits and one nephrectomized dog the decline in the concentrations of inulin and  $\text{SCN}^-$  in plasma

was traced after intravenous injection of a single dose of inulin + NaSCN.

It was found that:

1) The experimentally determined curve of distribution for SCN<sup>-</sup> (the volumes of distribution plotted against time) was found to be placed well above a theoretical curve of distribution for SCN<sup>-</sup>, constructed from the assumptions that inulin and SCN are finally distributed in the same volume and that this distribution proceeded exclusively by diffusion. The quantity of fluid in the erythrocytes is entirely insufficient to explain this discordance; it is presumably that SCN<sup>-</sup> penetrates other cells of the organism.

2) The distribution after a very long experimental period (12 hours) suggests that the ultimate distribution in rabbits will be such that inulin distributes itself in slightly less than 20 % of the body weight and SCN<sup>-</sup> in a little over 30 %.

3) In a few of the experiments, when the distribution of SCN<sup>-</sup> and inulin was well advanced, 100—120 ml. isotonic sulphate solution was infused into the rabbits. From the resulting dilution of plasma-chloride the author calculated the "volume of dilutable chlorides"; at a certain time after the sulphate infusion it was found to be less than the volume of distribution for SCN<sup>-</sup> at the same period of time after the NaSCN injection.

4) Whereas the available spaces for SCN<sup>-</sup> and Cl<sup>-</sup> must be supposed to be larger than the extracellular space, inulin must be assumed to distribute itself in a volume that is equal to the extracellular space. Tests showed that inulin is not excreted in rabbit bile, and experiments with blockade of the reticulo-endothelial system seem to indicate that inulin is not stored by this system.

5) Inulin's slow distribution, especially in sparsely vascularized tissue such as tendons, is, however, a serious drawback to its practical application for the determination of the extracellular space, even in nephrectomized animals.

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## The Significance of Diffusion and Convection for the Distribution of Solutes in the Interstitial Space.

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According to STARLING's wellknown theory the balance between the plasma fluid and the fluid in the interstitial spaces is governed by the following two factors: 1) the difference between the hydrostatic pressure within the capillaries and in the tissues, 2) the effective colloid-osmotic pressure of the plasma.

If 1 exceeds 2 in some capillaries or in the arterial end of the capillaries while 2 exceeds 1 in other capillaries or in the venous end of the capillaries the conditions for a flow: capillaries — interstitial spaces — capillaries are fulfilled. Such a flow must inevitable carry with it solutes for which the capillary wall is permeable, *i. e.* involve a convection of such solutes in the interstitial spaces.

However, different views have been held on the question whether the conditions for such a "convection-flow" are fulfilled in the organism.

SCHADE in 1927 sums up the results obtained by himself and his collaborators in experiments on models of artificial capillaries concluding that these experiments make it probable that in the natural capillaries a flow of fluid out of the arterial end and into the venous end of the capillaries occurs.

Judging from the measurements of the capillary blood pressure and the colloid-osmotic pressure of the plasma then in hand, KROGH in 1929 considers such a flow unlikely.

Nevertheless, the following, later experiments support the assumption that conditions for such a flow are fulfilled: LANDIS (1930 a) on the capillary blood pressure in the mesentery of mammals, LANDIS (1930 b) on the capillary blood pressure in human skin and KROGH, LANDIS and TURNER's (1932) pressure plethysmographic determinations of the variations in tissue volume accompanying 1) increase in venous pressure and 2) increase in colloid-osmotic pressure of plasma.

This is also the conclusion drawn by PETERS (1935) in his monograph "Body Water", p. 54.

Thus at present it seems justifiable to assume that such a flow of fluid through the interstitial spaces — "a paracapillary circulation" — occurs, inevitably involving a convection of capillary permeable solutes.

It is, however, a question whether this convection is significant for the to- and fro-exchange of solutes between blood and interstitial fluid and for their further distribution through the interstitial spaces or whether this exchange is mainly due to diffusion. This question, which apparently has not previously been tested experimentally is the main problem of the present paper.

### Principles Underlying the Experimental Procedure Employed.

In an earlier paper (KRÜHØFFER 1945 b) a number of arguments were presented in favour of the assumption that inulin is distributed in and exclusively in the extracellular space. The formula:  $\frac{\text{amount injected}}{\text{concentration in plasma water}}$  was used for the calculation of the volume in which inulin is distributed at any moment after the injection. The volume calculated according to this formula is named "*volume of distribution*".

The volume of distribution comprises plasma water, at first a part of it but quickly the whole of it. That part of the volume of distribution which is situated extravascularly will in the following be denominated "*volume of interstitial distribution*".

The model shown in Fig. 1 may be a help to visualize the effect of convection respectively diffusion on the curves of distribution of two non-electrolytes possessing different rates of diffusion as for instance inulin and sucrose. The model has two compartments

P representing plasma and I representing the interstitial space. The compartments are separated by a membrane permeable for inulin and sucrose. P is filled with water which is mixed very rapidly by stirring (ideal convection).

First suppose I to be a gel in which no convection can take place. At zero time certain amounts of inulin and sucrose are added simultaneously to P. Extremely rapidly the two solutes are evenly distributed in P and next they are distributed in I exclusively by diffusion. In this ease it is possible by means of the experimentally determined curve of interstitial distribution for

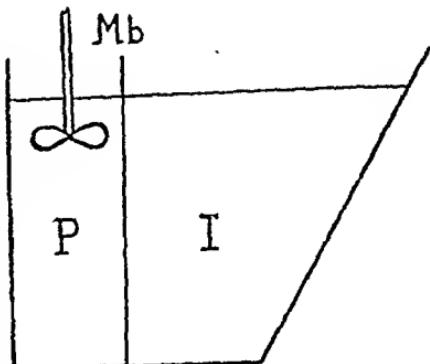


Fig. 1.

diffusion coefficient for sucrose  
inulin and the ratio:  $\frac{\text{diffusion coefficient for sucrose}}{\text{diffusion coefficient for inulin}} = n$  to construct a theoretical curve of interstitial distribution for the sucrose. The interstitial volume of distribution for sucrose at the time  $t$  will in this ease be equal to the determined interstitial volume of distribution for inulin at the time  $t \cdot n$ .

Then suppose I filled with water and two apertures in the membrane through which water is streaming in and out from P owing to the stirring of the water. If this flow of fluid is infinitely rapid in proportion to the rates of diffusion of the two solutes the distribution is effected solely by convection and their experimentally determined curves of interstitial distribution will be identical.

Finally suppose that the distribution of the two solutes is effected by convection as well as diffusion. Then the experimentally determined curve of interstitial distribution for sucrose will be situated between the experimentally determined curve of interstitial distribution of inulin and the theoretical curve of interstitial distribution of sucrose herefrom calculated.

The reason why inulin and sucrose in the above considerations have been chosen as instances is, that these two nonelectrolytes are suitable for analogous animal experiments as they possess the following properties:

- 1) When distribution is complete they are distributed in the same (extracellular) volume.

2) Their rates of diffusion differ considerably.

3) The elimination of both can be abolished by nephrectomy.

Two preliminary experiments have shown that sucrose 5 hours after injection in nephrectomized rabbits was distributed over respectively 17.5 and 19 % of the body weight. This result is in good agreement with the final volume of distribution for inulin found in earlier experiments (KRÜHØFFER 1945 b).

In the *animal body* the distribution in plasma from a practical point of view is brought about exclusively by convection, but contrary to our model in which the mixing occurs with infinite rapidity, the mixing takes some time involving that a certain diffusion into the interstitial space will have taken place before the distribution in the plasma is complete. For this reason determinations of the volume of interstitial distribution in the animal body are not quite so simple as in our model.

By injecting the dye T 1824 simultaneously with inulin and sucrose it is possible to determine the volume of plasma water with which the injected solution at any given time has been mixed (the mixing volume). The interstitial volume of distribution for inulin (respectively sucrose) is then calculated as: volume of distribution for inulin at a given time minus the mixing volume at the same time.

### Technique.

A technique corresponding to that published in a previous paper (KRÜHØFFER 1945 b) has been employed.

In the course of 15 seconds a single dose of inulin + sucrose + T<sub>1824</sub> was injected intravenously on nephrectomized rabbits (about 3 kg) in amytal anaesthesia. 200 mg of inulin + 350—400 mg of sucrose + 2 mg T<sub>1824</sub> dissolved in 10 ml 0.8 % NaCl were injected.

To reduce the error on the determination of sucrose (see below) the plasma glucose concentration was kept low during the experimental period by means of an intramuscular injection of 3—4 i. U. of insulin about one hour before the beginning of the experimental period.

In plasma from blood stabilized with heparin the concentrations of inulin, sucrose and glucose were determined.

T<sub>1824</sub>: The dilution of this dye was determined at different points of time after the injection by means of a HAVEMANN's (1940) photoelectric colorimeter. 0.5 ml of plasma from arterial blood was diluted with 4 ml of a 0.9 % NaCl-solution. The readings were made in a "Kleinkuvette" with a layer thickness of 20 mm using a yellow filter (GG<sub>1</sub>). For zero readings a solution containing 0.5 ml plasma drawn before the injection and 4 ml 0.9 % NaCl-solution was employed.

The degree of dilution was read from a standardization curve. For the construction of this curve different dilutions of  $T_{125}$  in 0.9% NaCl-solution were mixed with plasma in the same proportion as in the samples.

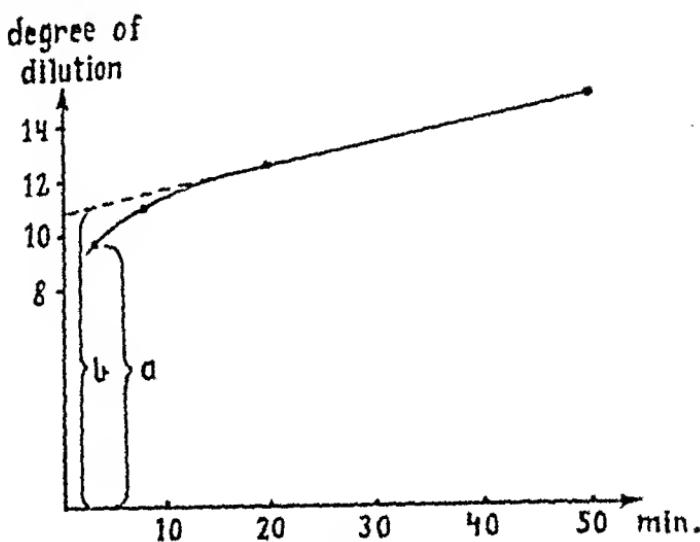


Fig. 2.

The curve Fig. 2 shows the variations in the degree of dilution of  $T_{125}$  during an experiment. Extrapolation to zero time has been made according to the modus operandi now prevalent in plasma volume determinations, only as the ordinate the degree of dilution was substituted for the concentration of dye. If mixing had occurred instantaneously the degree of dilution at zero time would have been 10.8, increasing from this point following the straight line, the incline of which denotes the extravascular loss of  $T_{125}$ . Hence, the volume of plasma water in the experiment presented was:  $10 \cdot 10.8 \cdot \frac{95}{100}$  ml.

The mixing volume for instance at time 3 minutes was:

$$10 \cdot 10.8 \cdot \frac{95}{100} \cdot a$$

$\frac{95}{100} b$ .

Determination of inulin and sucrose in plasma containing glucose.

As all methods for determination of sucrose proved to be sensitive also to inulin and glucose it was necessary to work out a special technique. This technique is based on the previously published colorimetric method for determination of inulin (Kuenenren 1945 a) in which the colour reaction on heating for 60 minutes to 100° C with resorcin dissolved in ethylalcohol and hydrochloric acid is made use of.

In this reaction glucose and sucrose give rise to a development of the same yellow colour as inulin, but the intensity of colour developed

differs for the same weight of the three substances as will be seen from table 1.

Table 1.

Glucose-conc. (mg %)	equiv. to inulin (mg %)	Thus, 1 mg inulin is equiv. to mg glucose	Sucrose-conc. (mg %)	equiv. to inulin (mg %)	Thus, 1 mg inulin is equiv. to mg sucrose
8.125	0.66	12.3	3.0	1.70	1.765
16.25	1.27	12.8	4.5	2.53	1.780
24.375	1.92	12.7	6.0	3.38	1.775
32.5	2.58	12.6	9.0	5.04	1.785
65.0	5.11	12.7	12.0	6.74	1.780
			18.0	10.16	1.775

From table 1 it can be deduced that in respect to colour development:

1 mg of inulin is on an average equivalent to 12.65 mg of glucose.

1 mg of inulin is on an average equivalent to 1.78 mg of sucrose.

The determination of sucrose in solutions containing inulin and glucose is based on the following three figures:

1. Total colour development expressed as mg % inulin.

2. Colour development after fermentation *i. e.* inulin concentration.

3. Reduction power determined after HAGEDORN and JENSEN (1923) which gives the glucose concentration as neither inulin nor sucrose reduces  $K_3FeCN_6$ . From the glucose concentration the colour development originating from glucose is calculated by means of the factor deduced from table 1 and expressed as mg % inulin.

By subtracting inulin plus glucose (expressed in the above mentioned way) from the total colour development, the colour development originating from sucrose is found. From this figure the concentration of sucrose is calculated by means of the sucrose factor from table 1. In this calculation allowance must be made for the loss of inulin which takes place by the removal of glucose and sucrose by fermentation. The recovery percentage by this procedure has experimentally been found to be 94. The figure for the inulin concentration therefore must

be multiplied by  $\frac{100}{94}$ . The recovery percentage by determination of the

total colour development and the reduction power on Somogyi filtrates is 100.

The analytic procedure described in detail in a previous paper (KRÜHoffer 1945 a) has been employed. Only to obtain a complete removal of sucrose as well as glucose by fermentation a somewhat larger amount of yeast (4 ml of a 10 % suspension for about 7 ml filtrate) had to be used, hence the lower recovery % (94) for inulin.

The accuracy of the sucrose determinations is of course highest when the concentrations of inulin and glucose are as low as possible. As mentioned above, the glucose concentration was kept low during the experimental period by means of insulin. The inulin concentration must be kept within 35 to 90 mg % to obtain the highest possible ac-

curacy in the inulin determinations. Only moderate sucrose concentrations can be used to avoid undesirable osmotic effects.

The following example shows the accuracy obtained at the concentrations used in the experiments: To a plasma containing 103 mg % glucose inulin was added to a concentration of 40 mg % and sucrose to a concentration of 75 mg %. In 5 determinations the following concentrations were found: Inulin 39.7; 39.9; 40.3; 40.4; 40.5 and sucrose: 73.9; 75.4; 73.6; 72.4; 72.1. At concentrations of this order of magnitude inulin apparently is determined with an accuracy of 1—2 % and sucrose with an accuracy of 3—4 %.

*Determination of the ratio between the diffusion coefficients for sucrose and inulin.* This determination was made using the same technique and the same diffusion chamber as described in a previous paper (KRUHOFER 1945 b). At 37° C the ratio  $D_{\text{sucrose}} / D_{\text{inulin}}$  was found to be 2.98. (The concentrations used in the inner fluid were 1,500 mg % inulin and 1,000 mg % sucrose.)

*Calculations:* The vo-

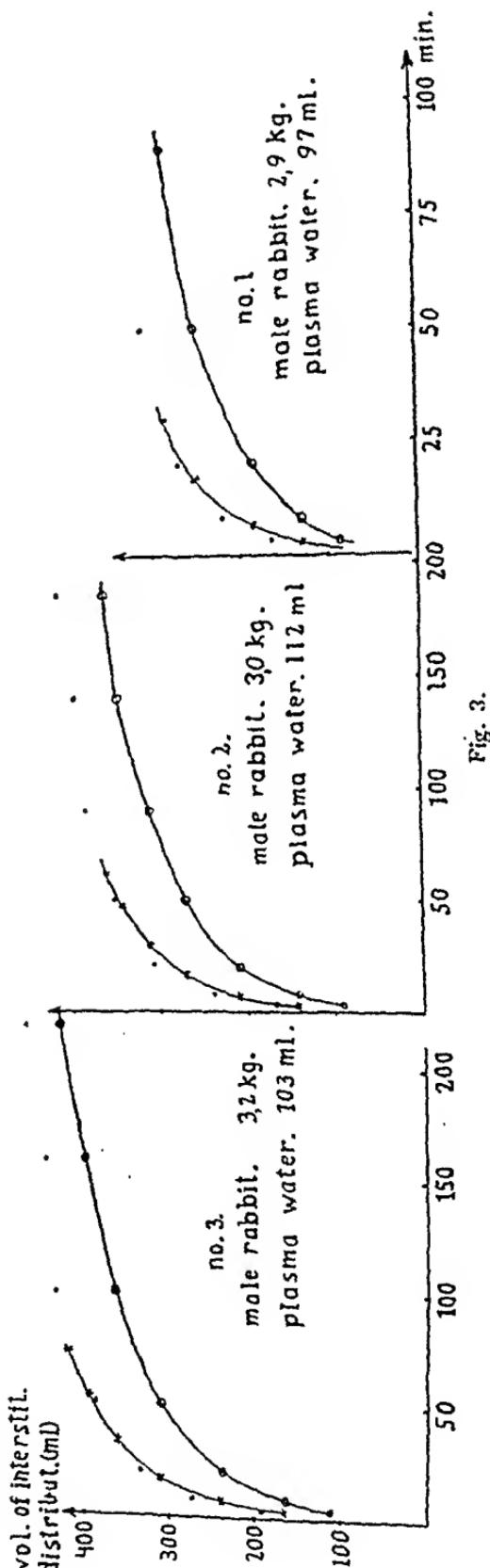


Fig. 3.

volumes of distribution for inulin and sucrose have been calculated according to the formula:

$$\frac{\text{amount injected}}{\text{concentration in plasma water}}$$

The concentration in plasma water was calculated as plasma concentration  $\frac{100}{95}$ .

### Experimental Results.

In Fig. 3 A the results from an experiment are presented graphically. The interstitial volumes of distribution (ordinates) are plotted against time (abscissa). The lowest curve ( $o - o$ ) represents the experimentally determined curve of interstitial distribution for inulin. The upper curve ( $x - x$ ) is the theoretical curve of interstitial distribution for sucrose calculated from the inulin curve by multiplying the abscissae from this curve with  $\frac{1}{2.98}$ .

The dots ( $\cdot$ ) which represent the experimentally determined values for the interstitial volumes of distribution for sucrose are seen to fall close to the theoretical curve.

In fig. 3 B and 3 C the results from two corresponding experiments are shown. A fourth experiment, not pictured, gave quite similar results.

### Discussion.

From fig. 3, it is seen that the experimentally determined values for the interstitial volume of distribution for sucrose on the whole fall close to the theoretically calculated curve. In accordance with the considerations made in the first part of this paper, it consequently must be assumed that diffusion dominates the distribution of solutes in the interstitial spaces.

All the experiments have in common that the experimentally determined volumes of interstitial distribution for sucrose in the first part of the experiments (about 20 minutes) are larger than the theoretically calculated values. In a simple, rigid system as the model fig. 1 such results could never be obtained.

This discrepancy might be explained in one of the following ways:

- 1) In the organism the ratio  $\frac{D_{\text{sucrose}}}{D_{\text{inulin}}}$  might be higher than that found in the experiments with the diffusion chamber. Here three phases must be considered: the capillary membranes, that part of

the interstitial fluid which is placed intrastructurally (within the collagenous fibres, etc.) and the remaining part placed extrastructurally.

As regards the last named, all the molecules it contains must be considered freely movable, and the viscosity must be almost the same as that of pure water. Consequently, in the extrastructurally placed part of the interstitial volume the diffusion rates of the different solutes must be almost the same as those found in pure water.

Concerning the two other phases, the capillary membranes and the intrastructural part of the interstitial space, it must be pointed out that both are built up of fixed molecules, which confer to them a definite molecular pattern. Beyond doubt, the rate by which solutes find their way through such a pattern will depend on their molecular size, the pattern offering more resistance to the progress of larger molecules. We have no definite conception of the composition and viscosity of the fluids filling out the lacunae in these patterns and thus we have no knowledge of the degree to which they affect the rates of diffusion for inulin and sucrose.

Summing up, it seems most probable that the ratio  $\frac{D_{\text{sucrose}}}{D_{\text{inulin}}}$  for the distribution within these two phases should be somewhat higher than in the case of pure aqueous solutions.

Compared to the extrastructural part of the interstitial volume, the intrastructural part and the capillary membranes possess only a small volume and further the intrastructural part is that most remote from the capillaries. Therefore, I think a higher  $\frac{D_{\text{sucrose}}}{D_{\text{inulin}}}$  ratio in the two structural phases cannot be the sole explanation of the discrepancy.

2) A living animal is not a rigid system as the model fig. 1.

If the membrane separating P and I during the first part of the experimental period were larger than later on, the experimentally determined interstitial volumes of sucrose would be somewhat larger than the corresponding calculated values until complete distribution is attained. The same thing would happen in the living animal if the number of open capillaries was larger during the first part of the experiment than later on. A successive closure of capillaries during the experiment, caused by a developing post-operative shock does not seem improbable and further it cannot be excluded that the rapid, intravenous injection of 10 ml fluid induces a temporary opening up of capillaries.

Certainly too, the spontaneous variations in the number of open capillaries may affect the total size of capillary membrane separating at any given moment plasma from interstitial fluid. However, as these variations must be supposed to occur at random about a rather fixed mean value they can hardly affect the results in a particular direction.

On the other side it is very difficult to estimate the net influence which the opening-closure intermittence of the capillaries may have on the course of the distribution curve; because this influence must be composed of more factors affecting the results in different ways.

By calculating the results in the manner mentioned above a systematic error is made. Owing to the more rapid distribution of sucrose a smaller percentage of this than of inulin will be removed in each blood sample drawn. Calculating the distribution volumes from the total amounts injected will thus result in estimating all volumes of distribution for inulin *relatively* too high (absolutely both are calculated a little too high). However the errors introduced in this way are only of limited consequence. (Yet eventual further experiments with the technique here suggested ought to be performed on larger experimental animals to make the blood samples represent only a minute fraction of the total blood volume.)

Though the technique here suggested thus involves certain uncontrollable sources of error when it is employed on the living animal, yet I think the above conclusion that diffusion completely dominates the distribution of sucrose and inulin in the interstitial spaces valid. That convection in some (quantitatively little important) tissues, particularly in tissues (like tendons, cornea etc.) with poor vascularization (long ways of diffusion), may play a certain rôle cannot of course be excluded. To decide whether this is actually the case determinations of the rates of distribution for inulin and sucrose in these particular tissues should be carried out, keeping the plasma concentrations at a constant level throughout the experimental period.

The diffusion rates of sucrose and particularly inulin are lower than the diffusion rates of some materials normally transported (glucose, urea etc.), consequently convection plays a still smaller part in the distribution of these materials in the interstitial spaces.

Finally the attention shall be drawn to the fact that from the renal physiology we know cells which can apparently be supplied with certain solutes by diffusion against a fluid stream; viz. the

cells of the proximal tubules which are able to receive considerable amounts of phenol red, diodrast etc. from the capillaries in spite of a fluid stream in the opposite direction through the interstitial spaces. (WALKER *et al.* (1941) demonstrated that large amounts of water must be reabsorbed in tubuli proximales.)

### Summary.

In experiments on nephrectomized rabbits inulin and sucrose have been found to have at least very nearly the same definite volume of distribution viz. slightly less than 20 % of the body weight. Both materials must be assumed to distribute themselves only in the extracellular space.

As sucrose diffuses about 3 times as rapidly as inulin, sucrose is more suitable for determinations of the extracellular space and must at present be considered the most appropriate substance for such determinations on nephrectomized animals and individual tissues, however complete distribution is not obtained till 4—6 hours after intravenous injection.

An experimental procedure is described which makes it possible to decide whether convection or diffusion is the most important factor in the distribution of solutes in the interstitial spaces. The method is based on determinations of the so called curves of distribution for inulin and sucrose after simultaneous intravenous injection on nephrectomized rabbits.

From the results of experiments of this kind it is concluded that diffusion completely dominates the distribution.

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# On Acute Effects of Cigarette Smoking on Oxygen Consumption, Pulse Rate, Breathing Rate and Blood Pressure in Working Organisms.

By

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A considerable number of papers on the effect of smoking can be found in the literature. The authors agree that in resting conditions there is a rise in pulse rate and blood pressure as a result of smoking. As to the effect on metabolic rate the opinions differ. About the effects of tobacco on a working organism we were, unfortunately, unable to find any works in literature. From the point of exercise physiology this, however, should be of particular interest. It is known that the metabolic rate and other functions show manifold rise in a working organism and that certain drugs and hormones have quite different actions in a resting and in a working organism. Therefore, by making experiments on organisms at the state of basic metabolism we cannot draw conclusions to the working organism.

The following experiments, inspired by prof. E. H. CHRISTENSEN, try to give some experimental basis to suggestions of the action of tobacco on working human organism.

## Methods.

The experiments were made with the Krogh's bicycle ergometer on which it is possible to exactly determine the amount of work done. A metronome was used for the timing of work controlling the rhythm of

pedalling (60 turns per min.). The turns of the bicycle were rerecorded by a cyclometer.

The rate of work varied in different experiments and has been recorded in the description of the experiments. Analyses of expired air were made by Haldane's apparatus for gas analysis. For the measuring of blood pressure Riva-Rocci's sphygmomanometer was used. Pulse rate was measured on A. earotis.

The determinations of respiratory functions, oxygen consumption, pulse rate and blood pressure at the state of rest for the purpose of control and comparison were made after 45 minutes' full rest. A. J., one of the subjects, even slept in the laboratory. After rest the subjects smoked, males two and females one cigarette, and 5 minutes after smoking (to eliminate the effect of movements in connection with smoking) new determinations were made. For smoking the same brand of cigarettes (Hudson) was used every time.

Before the exercise test the subjects rested for at least 30 minutes after which time pulse rate and blood pressure were measured. Then the subjects smoked and five minutes after smoking a new determination of pulse rate and blood pressure was made. Immediately exercise test followed. These test exercises were made alternately, one day with smoking, one day without, in order to eliminate the effects of training. Before the experiments the subjects fasted and did not smoke at least for 12 hours.

### Experimental Results.

The first experiments were made on male subject L. M., 30 years, 77.6 kg, 185 cm, moderate smoker (10—12 cigarettes daily) during the time of 8. II. 45—12. VI. 45. The subject, an active sportsman, was at the beginning of the experimental period untrained. The mean values and standard error of the mean  $e$  ( $M$ ) are brought in the tables.

From the experiments made at the state of basic metabolism (Table 1) we can see that there is a marked difference in pulse

Subj. L. M.

Table 1.

	Number of exp.	Vent. $37^{\circ}\text{l}/\text{min.}$	Breath. rate	$\text{O}_2 \text{l}/\text{min.}$	Pulse	Blood pressure	
						syst.	diast.
Rest-experiments.	no smoking $e$ ( $M$ )	3	6.62	6.7	0.265	60.7	108.3
	smoking $e$ ( $M$ )	3	$\pm 0.23$	$\pm 0.8$	$\pm 0.002$	$\pm 1.7$	$\pm 4.4$
			6.61	7.0	0.271	68.3	120.0
			$\pm 0.26$	$\pm 0.6$	$\pm 0.008$	$\pm 2.4$	$\pm 2.9$
							$\pm 0.0$

$e$  ( $M$ ) = Standard error of the mean.

rates. After smoking the pulse rate is faster than without smoking. A definite influence can also be noticed between the corresponding blood pressures; after smoking the blood pressure increases.

The first rate of work was 1,080 mkg per minute in 45 minutes (Table 2). The respiratory functions have been determined every 15, pulse rate every 5 and blood pressure every 10 minutes. Definite changes were obtained only in pulse rates. There is a rise also in blood pressure which, however, is not statistically certain.

The next rate of work was 1,260 mkg per minute in 30 minutes (Table 3). Respiratory functions were checked here as well as in following rates after every 10, pulse rate after 5 and blood pressure after every 7 minutes. Here we can also see an increase only in pulse rate, whereas other functions show no statistically significant changes.

Applying 1,440 mkg per minute in 20 minutes we see the same changes (Table 4). Sometimes even the pulse rate shows a statistically uncertain increase, particularly at the 5th minute. At the 20th minute we can again see a pulse increase of 18.7 beats per minute,  $e(M)$  being  $\pm 5.2$ .

The last rate of work was 1,560 mkg per minute in 10 minutes (Table 5). During these experiments the subject reached the limit of his capacity. He found it difficult to keep pace and complained over weariness which could also be ascertained objectively. Even at this rate of work the effect of smoking can be recognised only in changes of pulse rate.

At the end of these experiments pulse rate and blood pressure were measured after 5 minutes' rest and even here we can see that after the experiments with smoking the pulse does not reach the same low level after 5 minutes of rest as after the experiments without smoking.

The next experiments were made with A. J., male subject, 29 years, 85 kg, 183 cm, light smoker (3—4 cigarettes daily), well-trained.

In the state of basic metabolism we saw similar changes, there was a definite rise in pulse rate while blood pressure showed only a small rise which could not be regarded as statistically certain.

Exercise experiments were made on him with 1,260 mkg per minute in 30 minutes (5 experiments with and 5 without smoking). 1,440 mkg per minute in 20 minutes, 1,620 mkg per minute in 10 minutes and 1,800 mkg per minute in 10 minutes. With the last

## ACUTE EFFECTS OF CIGARETTE SMOKING.

Table 2.

		Min. of work				10 min.				15 min.				
		Breath.	O <sub>2</sub> l/min.	5 min. Pulse		Pulse syst.		Blood press.	Pulse		Pulse syst.		Blood press.	Pulse
Number of exp.	Vent. 37°l/min.													
no smoking	6	61.06	14.8	2.59	133.0	143.7	147.5	73.3	144.8	142.3	152.5	156.4	72.5	
o (M)	6	± 1.55	± 0.2	± 0.04	± 2.6	± 1.8	± 2.8	± 1.7	± 2.3	± 3.2	± 3.2	± 2.0	± 1.5	
no smoking	6	61.06	14.8	2.59	145.8	149.6	152.5	75.0	156.4	161.0	155.0	155.0	71.0	
o (M)	6	± 1.55	± 0.2	± 0.02	± 2.0	± 1.7	± 2.0	± 1.3	± 2.0	± 2.3	± 2.1	± 1.8	± 1.5	
15 min. smoking		Min. of work				30 min.				30 min.				
		Number of exp.				20 min.				25 min.				
		Vent. 37°l/min.	Breath. O <sub>2</sub> l/min.			Pulse syst.		Blood press.	Pulse	Pulse syst.		Blood press.	Pulse	
no smoking		6	61.70	15.20	2.57	145.5	148.0	71.0	147.3	153.5	152.5	152.5	72.5	
o (M)		6	± 1.07	± 0.02	± 0.03	± 1.8	± 3.8	± 1.0	± 2.4	± 3.2	± 3.2	± 3.2	± 1.5	
no smoking		6	61.70	15.20	2.57	155.7	155.8	72.5	158.8	161.0	155.0	155.0	71.0	
o (M)		6	± 1.07	± 0.02	± 0.03	± 1.8	± 1.9	± 2.1	± 1.8	± 2.7	± 2.7	± 2.7	± 1.8	
no smoking		Min. of work				After 5 min. rest.				After 5 min. rest.				
		Number of exp.				40 min.				45 min.				
		Vent. 37°l/min.	Breath. O <sub>2</sub> l/min.			Pulse syst.		Blood press.	Pulse	Pulse syst.		Blood press.	Pulse	
no smoking		6	62.02	15.0	2.63	150.7	151.5	150.8	70.0	156.8	156.3	111.0	75.0	
o (M)		6	± 0.71	± 0.0	± 0.02	± 3.4	± 3.0	± 2.8	± 0.0	± 2.5	± 4.7	± 3.0	± 1.6	
no smoking		6	61.51	15.5	2.65	161.5	162.8	151.3	72.5	165.8	165.3	113.0	78.0	
o (M)		6	± 1.0*	± 0.3	± 0.3	± 2.3	± 2.0	± 1.8	± 2.1	± 2.1	± 3.9	± 1.8	± 1.1	
45 min. smoking		1,080 mgs/ min.				30 min. smoking				30 min. smoking				
		Number of exp.				35 min.				35 min.				
		Vent. 37°l/min.	Breath. O <sub>2</sub> l/min.			Pulse syst.		Blood press.	Pulse	Pulse syst.		Blood press.	Pulse	
no smoking		6	62.02	15.0	2.63	150.7	151.5	150.8	70.0	156.8	156.3	111.0	75.0	
o (M)		6	± 0.71	± 0.0	± 0.02	± 3.4	± 3.0	± 2.8	± 0.0	± 2.5	± 4.7	± 3.0	± 1.6	
no smoking		6	61.51	15.5	2.65	161.5	162.8	151.3	72.5	165.8	165.3	113.0	78.0	
o (M)		6	± 1.0*	± 0.3	± 0.3	± 2.3	± 2.0	± 1.8	± 2.1	± 2.1	± 3.9	± 1.8	± 1.1	

\* (M) = Standard error of the mean.  
o (M) = Standard error of the mean.

Table 3.

		Min. of work				Min. of work				Min. of work									
		Number of exp.		Breath. ratio	O <sub>2</sub> l/min.	5 min. Pulse		7 min. Blood press. syst. diast.		10 min. Pulse		20 min. Pulse		After 5 min. rest.					
no smoking 0 (M)	4	62.91 ± 1.10	14.60 ± 0.02	2.82 ± 0.11	136.0 ± 6.1	158.8 ± 5.5	76.3 ± 3.0	147.8 ± 2.3	154.0 ± 3.5	no smoking 0 (M)	4	62.91 ± 1.10	14.60 ± 0.02	2.82 ± 0.11	136.0 ± 6.1	158.8 ± 5.5	76.3 ± 3.0	147.8 ± 2.3	154.0 ± 3.5
	4	65.12 ± 0.92	14.80 ± 0.28	2.87 ± 0.01	150.5 ± 1.5	148.3 ± 6.6	73.8 ± 1.2	154.0 ± 3.5	154.0 ± 3.5		4	65.12 ± 0.92	14.80 ± 0.28	2.87 ± 0.01	150.5 ± 1.5	148.3 ± 6.6	73.8 ± 1.2	154.0 ± 3.5	
10 min. smoking 0 (M)	4	62.91 ± 1.10	14.60 ± 0.02	2.82 ± 0.11	136.0 ± 6.1	158.8 ± 5.5	76.3 ± 3.0	147.8 ± 2.3	154.0 ± 3.5	10 min. smoking 0 (M)	4	62.91 ± 1.10	14.60 ± 0.02	2.82 ± 0.11	136.0 ± 6.1	158.8 ± 5.5	76.3 ± 3.0	147.8 ± 2.3	154.0 ± 3.5
	4	65.12 ± 0.92	14.80 ± 0.28	2.87 ± 0.01	150.5 ± 1.5	148.3 ± 6.6	73.8 ± 1.2	154.0 ± 3.5	154.0 ± 3.5		4	65.12 ± 0.92	14.80 ± 0.28	2.87 ± 0.01	150.5 ± 1.5	148.3 ± 6.6	73.8 ± 1.2	154.0 ± 3.5	
1,260 mkg/min.	4	66.31 ± 1.79	15.7 ± 0.5	2.90 ± 0.06	158.8 ± 4.3	72.5 ± 3.2	148.5 ± 3.6	151.3 ± 2.1	159.5 ± 3.8	1,260 mkg/min.	4	66.31 ± 1.79	15.7 ± 0.5	2.90 ± 0.06	158.8 ± 4.3	72.5 ± 3.2	148.5 ± 3.6	151.3 ± 2.1	159.5 ± 3.8
	4	67.23 ± 2.51	15.9 ± 0.9	2.93 ± 0.01	156.3 ± 2.4	75.0 ± 0.0	155.0 ± 4.5	159.5 ± 3.8	159.5 ± 3.8		4	67.23 ± 2.51	15.9 ± 0.9	2.93 ± 0.01	156.3 ± 2.4	75.0 ± 0.0	155.0 ± 4.5	159.5 ± 3.8	159.5 ± 3.8
20 min. smoking 0 (M)	4	66.31 ± 1.79	15.7 ± 0.5	2.90 ± 0.06	158.8 ± 4.3	72.5 ± 3.2	148.5 ± 3.6	151.3 ± 2.1	159.5 ± 3.8	20 min. smoking 0 (M)	4	66.31 ± 1.79	15.7 ± 0.5	2.90 ± 0.06	158.8 ± 4.3	72.5 ± 3.2	148.5 ± 3.6	151.3 ± 2.1	159.5 ± 3.8
	4	67.23 ± 2.51	15.9 ± 0.9	2.93 ± 0.01	156.3 ± 2.4	75.0 ± 0.0	155.0 ± 4.5	159.5 ± 3.8	159.5 ± 3.8		4	67.23 ± 2.51	15.9 ± 0.9	2.93 ± 0.01	156.3 ± 2.4	75.0 ± 0.0	155.0 ± 4.5	159.5 ± 3.8	159.5 ± 3.8
no smoking 0 (M)	4	66.31 ± 1.79	15.7 ± 0.5	2.90 ± 0.06	158.8 ± 4.3	72.5 ± 3.2	148.5 ± 3.6	151.3 ± 2.1	159.5 ± 3.8	no smoking 0 (M)	4	66.31 ± 1.79	15.7 ± 0.5	2.90 ± 0.06	158.8 ± 4.3	72.5 ± 3.2	148.5 ± 3.6	151.3 ± 2.1	159.5 ± 3.8
	4	67.23 ± 2.51	15.9 ± 0.9	2.93 ± 0.01	156.3 ± 2.4	75.0 ± 0.0	155.0 ± 4.5	159.5 ± 3.8	159.5 ± 3.8		4	67.23 ± 2.51	15.9 ± 0.9	2.93 ± 0.01	156.3 ± 2.4	75.0 ± 0.0	155.0 ± 4.5	159.5 ± 3.8	159.5 ± 3.8
no smoking 0 (M)	4	66.31 ± 1.79	15.7 ± 0.5	2.90 ± 0.06	158.8 ± 4.3	72.5 ± 3.2	148.5 ± 3.6	151.3 ± 2.1	159.5 ± 3.8	no smoking 0 (M)	4	66.31 ± 1.79	15.7 ± 0.5	2.90 ± 0.06	158.8 ± 4.3	72.5 ± 3.2	148.5 ± 3.6	151.3 ± 2.1	159.5 ± 3.8
	4	67.23 ± 2.51	15.9 ± 0.9	2.93 ± 0.01	156.3 ± 2.4	75.0 ± 0.0	155.0 ± 4.5	159.5 ± 3.8	159.5 ± 3.8		4	67.23 ± 2.51	15.9 ± 0.9	2.93 ± 0.01	156.3 ± 2.4	75.0 ± 0.0	155.0 ± 4.5	159.5 ± 3.8	159.5 ± 3.8
30 min. smoking 0 (M)	4	66.31 ± 1.79	15.7 ± 0.5	2.90 ± 0.06	158.8 ± 4.3	72.5 ± 3.2	148.5 ± 3.6	151.3 ± 2.1	159.5 ± 3.8	30 min. smoking 0 (M)	4	66.31 ± 1.79	15.7 ± 0.5	2.90 ± 0.06	158.8 ± 4.3	72.5 ± 3.2	148.5 ± 3.6	151.3 ± 2.1	159.5 ± 3.8
	4	67.23 ± 2.51	15.9 ± 0.9	2.93 ± 0.01	156.3 ± 2.4	75.0 ± 0.0	155.0 ± 4.5	159.5 ± 3.8	159.5 ± 3.8		4	67.23 ± 2.51	15.9 ± 0.9	2.93 ± 0.01	156.3 ± 2.4	75.0 ± 0.0	155.0 ± 4.5	159.5 ± 3.8	159.5 ± 3.8

o (M) = Standard error of the mean.

Table 4.

	Number of exp.	Vent. 37°l/min.	Breath. ratio	$O_2$ l/min.	Min. of work			
					5 min. Pulse	7 min. Blood press. syst.   diast.	10 min. Pulse	
1,440 mkg/ min.	no smoking	3	73.23	16.5	3.19	147.3	158.3	73.3
	o (M)	3	± 1.74	± 1.3	± 0.04	± 8.7	± 1.7	± 3.3
	smoking	3	74.07	16.3	3.27	155.3	161.6	80.0
	o (M)	3	± 2.99	± 0.9	± 0.03	± 5.9	± 6.0	± 0.0

	Number of exp.	Vent. 37°l/min.	Breath. ratio	$O_2$ l/min.	Min. of work			
					14 min. Blood press. syst.   diast.	15 min. Pulse	19 min. Blood press. syst.   diast.	20 min. Pulse
20 min.	no smoking	3	75.66	16.6	3.30	161.7	169.7	163.3
	o (M)	3	± 1.80	± 0.3	± 0.03	± 3.3	± 6.4	± 2.9
	smoking	3	76.81	17.3	3.25	161.3	81.7	182.0
	o (M)	3	± 3.20	± 1.2	± 0.07	± 3.3	± 1.7	± 4.2

o (M) = Standard error of the mean.

Table 5.

	Number of exp.	Vent. 37°l/min.	Breath. ratio	$O_2$ l/min.	Min. of work			
					5 min. Pulse	7 min. Blood press. syst.   diast.	10 min. Pulse	After 5 min. rest. Blood press. syst.   diast.
1,560 mkg/ min.	no smoking	3	107.69	25.5	3.73	154.8	180.0	76.7
	o (M)	3	± 2.14	± 0.3	± 0.07	± 0.7	± 2.9	± 1.7
	smoking	3	104.76	25.3	3.69	162.7	181.7	81.7
	o (M)	3	± 3.33	± 0.7	± 0.08	± 2.4	± 1.5	± 1.5

o (M) = Standard error of the mean.

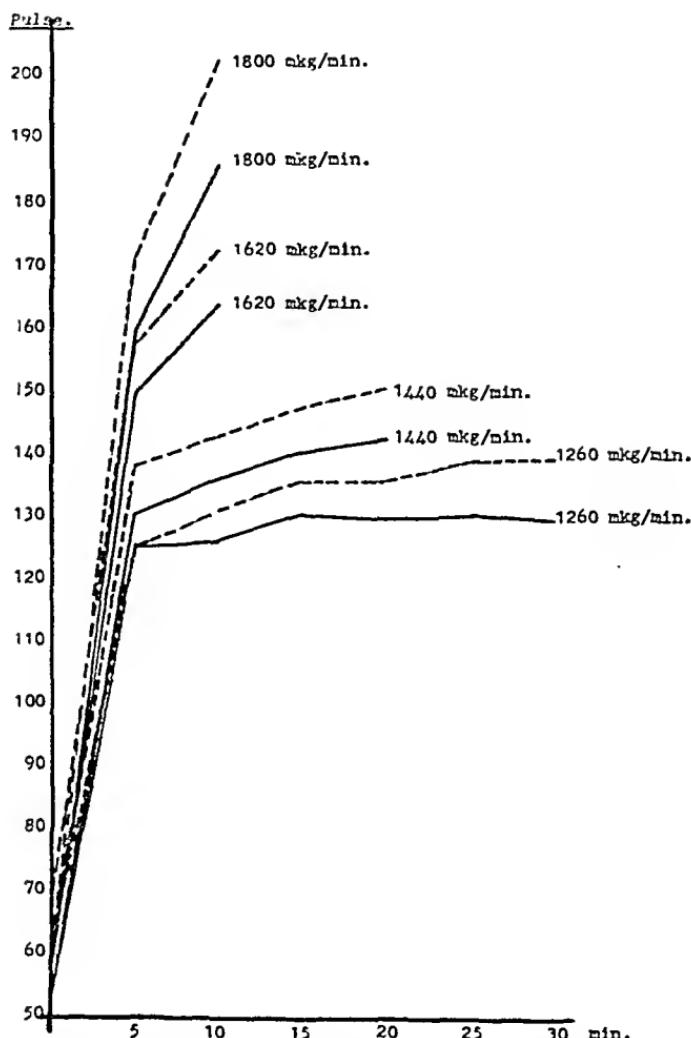


Fig. 1. Effect of smoking on pulse rate at different amounts of work. Subject A. J. —— smoking — no smoking. Each point represents the mean of 3 to 5 single determinations.

three rates of work 3 experiments were made with and 3 without smoking. The results showed that ventilation and breathing rate as well as the oxygen consumption are stable, showing no effect of smoking, whereas pulse rate again showed a definite rise. Here we could see once more that after 5 minutes the rest-pulse rate still being under the effect of smoking did not reach the same level as in other experiments made without smoking. Fig. 1 shows the effect of smoking on pulse rate at different amounts of work. Curve of the pulse rate after smoking runs at a higher level.

With the subject G. B. male, 25 years, 73.9 kg, 171 cm, well trained and non-smoker, only one series of experiments were made, namely with 1,620 mkg per minute in 10 minutes, 3 experiments were made with and 3 without smoking. Here roughly similar results were obtained. The subject did not feel well after smoking and was once compelled to finish the experiment for that reason. In pulse rate we could see a big difference after 5 minutes (20 beats per minute,  $e(M)$  being  $\pm 6.9$ ), that however diminished after 10 minutes (5 beats per minute,  $e(M)$  being  $\pm 7.1$ ) becoming statistically uncertain. The subject told by the way that the first 5 minutes after smoking had been the most strenuous, later on the driving had been easier.

The experiments made on female subject H. R., 28 years, 172 cm, 72 kg, light smoker (2—4 cigarettes daily) gave following results:

At the state of basic metabolism the results showed correspondence with the previous ones. There was a sure rise only in pulse rate and blood pressure. After smoking the subject complained of dizziness and nausea.

The first rate of work was 540 mkg per minute in 20 minutes (Table 6). Pulse rate shows no changes. The subject admitted that as she had not felt well at the previous experiments (at basic metabolism) she tried to smoke with caution this time. That could also be seen from the rise in pulse rate at rest. At the state of basic metabolism the rise in pulse rate was 13 beats, here only 8.

The fact that the pulse rate here does not give higher values at work depends on the short duration of the effect of tobacco in this subject. An experiment was made on her at the state of basic metabolism: when after a 30 minutes' rest she smoked one cigarette her pulse rate showed the resting-state values already at the 10th minute. Considering that the beginning of exercise test was actually 5 minutes after the smoking we can understand why the 5 minutes' pulse at work (*i. e.* ten minutes after smoking) does not show rise any more when compared with the pulse rate without smoking.

At the next experiment, 720 mkg per minute in 20 minutes (Table 7) the subject smoked 2 cigarettes instead of 1 at the previous experiment, trying to deeply inhale the smoke. She had nausea after each smoking, after two experiments she vomited (there were three experiments with and three without smoking). The results show a pronounced rise in ventilation and breathing

## Subj. H. R.

Table 6.

	Number of exp.	Vent. 37°/min.	Breath. ratio	$O_2$ l/min.	Min. of work			
					5 min.		7 min.	
					Pulse	syst.	Blood press.	diasst.
no smoking	4	25.27	6.5	1.38	137.5	144.3	68.8	139.5
		± 1.20	± 0.4	± 0.03	± 3.8	± 6.4	± 2.4	± 3.0
	10 min. smoking	26.71	7.1	1.15	135.5	149.0	71.3	139.5
o (M)	4	26.71	7.1	± 0.7	± 1.5	± 9.5	± 2.4	± 1.0
		± 1.45						
	o (M)							
540 mkg/ min.	Number of exp.	Vent. 37°/min.	Breath. ratio	$O_2$ l/min.	Min. of work			
					14 min.		19 min.	
					Pulse	syst.	Blood press.	diasst.
no smoking	4	25.28	6.8	1.43	150.0	67.5	143.0	147.5
		± 1.35	± 0.4	± 0.03	± 2.1	± 1.5	± 4.4	± 3.6
	20 min. smoking	27.09	7.1	1.50	156.3	68.8	143.0	152.7
o (M)	4	27.09	7.1	± 0.5	± 0.03	± 6.2	± 1.3	± 1.7
		± 1.00						
	o (M)							

o (M) = Standard error of the mean.

Table 7.

		Min. of work					
Number of exp.	Vent. 37 l/min.	Breath- rate	O <sub>2</sub> l/min.	5 min. Pulse	7 min. Blood pres. syst., diast.	10 min. Pulse	
no smoking	3	33.73	9.7	114.0	163.3	73.3	154.6
<sup>e</sup> (M)	$\pm 4.12$	$\pm 0.3$	$\pm 0.63$	$\pm 4.0$	$\pm 4.4$	1.3	$\pm 1.3$
10 min. smoking	3	44.79	14.0	152.7	146.7	73.3	155.3
<sup>e</sup> (M)	$\pm 1.54$	$\pm 0.6$	$\pm 0.63$	$\pm 3.7$	$\pm 6.0$	1.7	$\pm 2.9$
		Min. of work					
Number of exp.	Vent. 37 l/min.	Breath- rate	O <sub>2</sub> l/min.	14 min. Blood pres. syst., diast.	15 min. Blood pres. syst., diast.	20 min. Pulse	
no smoking	3	35.11	11.2	136.7	71.7	154.7	76.7
<sup>e</sup> (M)	$\pm 3.53$	$\pm 1.5$	$\pm 0.64$	$\pm 1.7$	$\pm 0.7$	1.7	$\pm 1.7$
20 min. smoking	3	46.56	15.9	163.3	73.3	160.7	73.3
<sup>e</sup> (M)	$\pm 2.31$	$\pm 0.6$	$\pm 0.61$	$\pm 1.7$	$\pm 1.7$	$\pm 4.3$	$\pm 1.7$
720 mkg/ min.		After 5 min. rest.					
		Blood pres. syst., diast.					
		Pulse					
		After 5 min. rest.					
		Blood pres. syst., diast.					
		Pulse					

<sup>e</sup>(M) = Standard error of the mean.

rate. Oxygen consumption is practically unchanged again. Pulse rate shows no rise.

Increased ventilation and breathing rate at 720 mkg can be taken as symptoms of acute nicotine poisoning. There were other symptoms: increased secretion of saliva, nausea, vomiting, cold sweat.

Lastly attempts were made to determine duration of the influence of smoking. Experiments were made on L. M. and A. J. After a rest of 15 minutes they smoked 2 cigarettes each which was followed by 30 minutes' sitting and a 30 minutes' lying. After that time (*i. e.* 60 min. after smoking) they made exercise test with 1,440 mkg per minute in 10 minutes. The results show that after 60 minutes there was no influence whatever of the smoking to be seen. After 45 minutes there was still a slight influence of smoking in L. M. The pulse rate was higher. In A. J. the influence was still noticeable after 30 min.

### Discussion.

As already mentioned we were unable to find any papers on the effect of smoking on the working organism. Quite a number of papers, however, could be found describing the influence of smoking on the organism at rest.

GOODMAN (1914) claims that smoking increases blood pressure by 10—30 mm. Hg. whereby the increase depends on the strength of tobacco.

FISHER and BERRY (1917) noted a rise in pulse rate together with rise in blood pressure whereas the changes were not characteristic.

SHORT and JOHNSON (1939) assert that changes in pulse rate, blood pressure, skin temperature and blood sugar can be explained by increased secretion of adrenaline. By injecting 1 cc. of 1 : 1,000 adrenaline solution they got similar results to those after smoking. They consider it probable that this is the stimulating influence of nicotine on the sympathico-adrenal system.

HIESTAND, RAMSEY and HALE (1940) found that smoking increases metabolism and pulse rate whereas breathing rate diminishes. These changes, however, do not correspond in all cases as there is an increase in metabolism in 82 per cent, a decrease in

13 per cent and no change in 5 per cent of all the subjects. There is an increase in pulse rate in 72 per cent, a decrease in 26 per cent and no change in 2.5 per cent of cases. The breathing rate showed rise in 41 per cent, fall in 53.9 per cent and no change in 5.1 per cent of cases.

MAIN (1941) finds that changes in pulse rate and blood pressure last for 30—60 minutes after smoking.

GODDARD and VOSS (1942) assert that basic metabolism may be increased or decreased by smoking depending on the sensibility of the person's sympathico-adrenal system.

STEINMANN and VOEGELI (1942) examined changes in circulation after their subjects had smoked and passed through a work-test. They found that after work the minute-volume was increased which was chiefly due to higher pulse rate whereas in the stroke volume there was either a small increase or decrease.

All those statements correspond with the results as to the changes in pulse rate and blood pressure under effect of smoking obtained in this work. The changes in oxygen consumption, however, make an exception in so far as there was no increase under the influence of smoking.

Although it is highly probable that smoking increases the secretion of adrenaline in the suprarenals, we can possibly not ascribe all the changes that have been recorded to the influence of this agent. The smoke of cigarettes, as we know contains nicotine as well as carbon monoxide, HCN, furfural, NH<sub>3</sub>, pyridine and its derivatives like pyridyl piperidin, collidin and nornicotine (an isomer of nicotine), quinoline, phenols, carbon dioxide and some volatile oils, which all in some way can influence the organism.

CHRISTENSEN (1931) finds that extended training decreases rest pulse as well as exercise pulse rate, both rates being lower in trained than in untrained subjects. The pulse rate in trained subjects reaches a certain level staying at this height until the end of the work. In untrained subjects the pulse rate rises continually during the work.

If we compare those data with the changes in pulse rate obtained in this paper we can see that smoking has a contrary effect to that of training on the pulse rate, viz. it deteriorates the condition.

### Conclusions.

- 1) Smoking calls forth a rise in pulse rate at the state of rest as well as at work.
- 2) Smoking has no effect on oxygen consumption.
- 3) It may be assumed that smoking does not affect ventilation and breathing rate.
- 4) Rise in blood pressure as effect of smoking is probable.
- 5) At a certain oxygen consumption, pulse rate is higher under the effect of smoking than no smoking.
- 6) The duration of the influence of smoking is 10—45 min., showing individual variations and depending on the amount of tobacco smoked.

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## Amino Acids and Related Compounds in the Haemolymph of *Oryctes Nasicornis* and *Melolontha Vulgaris*.

By

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One of the most characteristic features in the biochemistry of insect haemolymph is the high content of non-protein nitrogen (DUVAL, PORTIER *et al.* 1928). Indeed in most forms the greater part of the osmotic pressure comes from amino acids or related compounds.

Whereas amino nitrogen determinations on the haemolymph of different insect species have been made by several investigators, our knowledge concerning the nature of the amino nitrogen found is but scanty. HELLER (1930) found that the amino nitrogen of deproteinized *Deilephila*-blood increased considerably on acid hydrolysis, from which it was concluded that a considerable part of the amino-N originated from peptides. He further found that a considerable part of the amino-N could be precipitated by phosphotungstic acid and by uranylacetate.

FLORKIN and DUCHATEAU (1942) were unable to detect arginine, tryptophane, phenylalanine and cystine in the blood of *Dytiscus marginalis*. 30 mg% histidine and 117—168 mg% tyrosine were found, but this is quite insufficient to account for those 106 mg% amino-N, which the blood was shown to contain.

The question about the nature of the amino nitrogen of insect blood thus invited to further study. Larvae of *Oryctes nasicornis* and *Melolontha vulgaris* were chosen for the investigation. One result, the isolation of asparagine from *Melolontha* blood has been published in a note (USSING 1945 a).

One apparent implication of the high amino-N concentration in insect blood is that these animals, especially the herbivorous forms, have to take up amino acids from the intestinal contents against a high concentration gradient, and the insects might present a good object for the study of active amino acid uptake.

From the papers of HELLER (l. c.) we learn, however, that a considerable part of the amino-N originates from peptides in *Deilephila*-blood and FLORKIN and DUCHATEAU (l. c.) failed to find several important amino acids in *Dytiscus*, so it could not be taken for granted that a general mechanism for active amino acid uptake is at work in the intestine of insects. It might well be that the high amino-N content originated from one or some few amino compounds. In the course of the present study it was therefore decided to undertake some analyses on intestinal contents in order to allow a comparison between the concentrations of different amino acids in the blood and in the intestine.

## Experimental.

### 1. Preparation of protein-free blood extracts.

An incision was made in the side just behind the head of the larvae. The blood was allowed to fall directly into a measured amount of 20% trichloroacetic acid in a weighed vessel. When a suitable amount of blood — from several larvae — had been collected, the vessel was weighed and the volume was made up to a multiple of the blood volume: then the mixture was stirred and left at room temperature for one hour. The protein-free solution was filtered off into a measuring cylinder and then freed from trichloroacetic acid by extraction with ether in a continuous extractor. The ether was removed by aeration or concentration in vacuo.

### 2. Separation of the blood-filtrate amino compounds in three main fractions.

In order to get an idea of the distribution of the amino-N on the different amino-acid groups, blood filtrates were treated with phosphotungstic acid, which precipitates most peptides and the basic amino acids. After removal of excess precipitant the filtrates were saturated with  $\text{Ba}(\text{OH})_2$  and the dicarbonic acids precipitated with about 5 vol. alcohol. After removal of inorganic ions from precipitates and filtrates as well, three fractions resulted which are in the following designated as PW-fraction, Ba-fraction and monamino-acid fraction. The analysis on blood from *Melolontha*  $\frac{2}{3}$  may serve as an example:

The blood from 25 larvae, 7.1 g, was collected in 7 ml trichloroacetic acid. Water was added to the 70 ml mark and after mixing and standing for one hour, the solution was filtered off. Filtrate 64 ml. A drop of 10 % sulfuric acid was added and trichloroacetic acid removed by extraction with ether. The solution was concentrated in vacuo to a small volume and transferred with water in several portions to a cylinder. Volume: 20 ml. 5 ml drawn for analyses: the rest made 2.5 % as regards sulfuric acid and mixed with 1 g phosphotungstic acid, dissolved in a little hot water. After standing over night in the cold, the precipitate was centrifuged and washed with dilute phosphotungstic acid solution in 2.5 % sulfuric acid. The precipitate was suspended in water and decomposed with an excess of warm saturated  $\text{Ba}(\text{OH})_2$  and after removal of the precipitate and washing, the excess of  $\text{Ba}^{++}$  was immediately removed by addition of  $\text{H}_2\text{SO}_4$ : PW-fraction. The combined filtrates from the phosphotungstic precipitate were freed from  $\text{H}_2\text{SO}_4$  and excess precipitant by addition of warm saturated  $\text{Ba}(\text{OH})_2$  and the precipitate washed three times with hot water. The combined filtrates, 25 ml, were saturated with  $\text{Ba}(\text{OH})_2$  and mixed with 125 ml alcohol. After 18 hours in the cold, the precipitate was filtered off and washed with 85 % alcohol. The precipitate was freed from  $\text{Ba}^{++}$ , the  $\text{BaSO}_4$  being thoroughly washed with hot water: Ba-fraction. The alcoholic filtrate was concentrated in vacuo until all alcohol had been distilled off and then the  $\text{Ba}^{++}$  was removed quantitatively: mon-amino acid fraction.

Table I.

	Total N mg%	Amino-N mg%
Oryctes 1/2,		
Total blood .....	(424)	316
PW-fraction .....	181	67
Ba-fraction .....	22	17
Monamino acid fraction .....	250	180
Melolontha 1/2,		
Total blood .....	402	280
PW-fraction .....	94	52
Ba-fraction .....	17	15
Monamino acid fraction .....	160	129

Table I gives examples on the distribution of total N and amino-N in blood-filtrate from Oryctes and Melolontha. Total nitrogen is determined by micro-Kjeldal analysis and amino-nitrogen (after removal of ammonia by distillation in vacuo at pH 9) according to the modification of Folin's colorimetric method described by USSING (1945 b).

The value for total nitrogen on untreated filtrate from Oryctes is placed in brackets, because it refers to another blood sample than that used for the other analyses.

It will be noticed, that the sum of the amino-N of the three fractions from Oryctes gives 264 mg%, whereas the original amount was 316. In the case of Melolontha there is an even bigger loss of amino-N during the procedure. In order to locate the loss, an experiment was made on Oryctes blood where analyses were performed on the filtrate from the PW-precipitation, before the Ba-alcohol treatment. The results are shown in table II. It is seen that the calculated amino-N concentration of the PW-fraction is 38 mg% higher than that found. Probably the loss is at least partly due to adsorption of peptides to Ba-phosphotungstate which substance is known to adsorb certain peptides, for instance glutathione. Another important factor which leads to loss of nitrogen is the splitting of glutamine. We shall return to this point below.

Table II.

	Total N mg%	Amino-N mg%
Oryctes $\text{22/11}$		
Total blood .....	496	284
PW-fraction found .....	175	82
Filtrate from PW-fraction .....	232	164
PW-fraction calculated .....	264	120

The following conclusions seem to be valid for the blood amino-acids of both species studied: 1) The dicarbonic acids (the Ba-fraction) are quantitatively of little importance.

2) The PW-fraction may contain hexonbases and low peptides (di- and tri-peptides) but hardly higher peptides to any extent. This is easily seen by comparing total nitrogen and amino-nitrogen.

3) The monamino acid fraction contains more total nitrogen than amino nitrogen. This may mean, that this fraction contains amides or possibly low peptides which have not been precipitated by the phosphotungstic acid. Of course the excess nitrogen might originate from substances other than those showing amino acid reactions. One source of excess nitrogen in Melolontha has, however, been found already, namely asparagine.

### 3. The PW-fraction.

In order to see to what extent the PW-fraction consisted of peptides the following experiment was made: Half the PW-fraction from Oryctes  $\text{22/11}$  (10 ml) plus 1 ml conc. hydrochloric acid was evaporated in a test tube on a steam bath until only 2 ml remained. Then the tube was

sealed and kept at 100° for 18 hours. After that the tube was opened, the contents evaporated to dryness and the residue taken up in 10 ml water. After a sample had been drawn for amino-N determination the remaining solution was acidified with a drop of sulphuric acid and precipitated with phosphotungstic acid in small excess. (Now that the peptides are split phosphotungstic acid should only precipitate hexonbases.) The precipitate was freed from inorganic ions. The resulting solutions were used for amino-N determinations. The results were the following:

Calculated as mg amino-N  
per g original blood

PW-fraction ( $\frac{22}{11}$ ) before hydrolysis .....	0.82
»     »     » after     » .....	1.03
Hexonbases in hydrolysate .....	0.40
Non-Hexonbases in hydrolysate .....	0.51

It is seen that the amino-N increases 0.21 mg per g blood on hydrolysis; at the same time 0.51 mg N has appeared which is no longer precipitated by phosphotungstic acid. This indicates that the peptides in question are di- or tri-peptides. About half the amino-N in the PW-fraction comes from hexonbases.

A similar study was made on the PW-fraction of Melolontha blood ( $\frac{27}{10}$ ). A suitable part of this fraction was diluted to 5 ml. 0.5 ml from this solution contained ..... 0.655 mg amino-N. After hydrolysis a corresponding sample

contained ..... 0.692 mg amino-N.

In the remaining part of the hydrolysate histidine + arginine were precipitated as the Ag-salts and in the filtrate, freed from  $\text{Ag}^+$ , lysine was precipitated as the phosphotungstate.

Amino-N determinations gave the following results:

Histidine + arginine <sup>1</sup> .....	1.24 mg amino-N
Lysine-fraction .....	1.39     »     »
Filtrate from lysine fraction .....	0.53     »     »

It is seen that peptides contribute to a minor part only to the amino-N of Melolontha blood. As pointed out in section 2 some peptide may have been lost by adsorption on Ba-phosphotungstate.

From the histidine + arginine fraction histidine and arginine were fractionally precipitated as Ag-salts according to STEUDEL (1910). The histidine was lost. The arginine fraction was concen-

<sup>1</sup> The Folin method gives much too low values for arginine, whereas lysine reacts with both amino groups, thus giving per mole about twice the colour-value of that given by a monamino acid (FOLIN 1922).

trated to one ml at pH 4—5 and precipitated with 30 mg flavianic acid. The orange coloured arginine flavianate was twice recrystallized from one ml water containing a trace of flavianic acid. Each time three days in the cold were allowed for the crystallization.

Crop: 19.0 mg arginine flavianate, corresponding to 6.8 mg arginine.

The lysine fraction, freed from inorganic ions, was concentrated with a trace of BaCO<sub>3</sub> to a small volume. It was then freed from BaCO<sub>3</sub> by centrifuging and the solution was concentrated to about 100  $\mu$ l. Two vol. alcohol, followed by 20 mg picric acid in hot alcohol were added and the lysine picrate was allowed to crystallize in the cold.

Crop: 11.2 mg lysine pierate, corresponding to 4.4 mg lysine. It was left undecided if the lysine fraction contained putrescine, which substance together with lysine was found by ACKERMANN (1920) in an extract of whole Melolontha larvae.

From the above it follows that the greater part of the PW-fraction from Melolontha consists of the basic amino acids; arginine and lysine have been isolated and in chapter 7 it will be shown that histidine is also present in appreciable amounts.

#### 4. The Ba<sup>++</sup>-fraction.

From table I it is seen that the total N and amino-N of this fraction is about equal. The fraction therefore probably consists of monaminodicarbonic acids like aspartic and glutamic acids. Although the amount of amino-N in this fraction is rather low, the values are probably still too high. There is good evidence that some glutamic acid may have been formed from glutamine during the analyses.

Especially in Melolontha the Ba-fraction was rich in glycogen. On evaporation a glassy residue was left, which consisted mainly of this polysaccharid.

#### 5. Amides: Asparagine and glutamine.

As shown in table I the monamino acid fraction contains more total nitrogen than amino-N. To find out whether this excess N originated from amides the following hydrolysis was made:

4 ml monamino acid filtrate (Oryctes 31%), corresponding to 1.6 g blood and 1 g H<sub>2</sub>SO<sub>4</sub>, were kept at 100° for 18 hours in a sealed ampoule.

$H_2SO_4$ was removed with $Ba(OH)_2$ , and amino-N determinations were made before and after removal of ammonia	
before hydrolysis .....	0.75 mg N/g blood
after " "	0.90 " " "
" " and removal of ammonia ....	0.63 " " ?

It is thus clear that the increase in amino-N on hydrolysis is due to formation of ammonia. This made it probable, that the blood contains an amide. This suggestion was strengthened when it turned out, that the monamino acid fraction gave a precipitate with mercuric acetate. The experiments were continued on Melolontha blood from which asparagine was subsequently isolated. The procedure is described elsewhere (USSING 1945 a) together with the analytical data, which should not be recapitulated here.

Only a few points may deserve further mentioning: At first it seemed impossible, that the substance isolated should be asparagine, because it was found that the amino-N (determined colorimetrically) was only 36.5 % of the total N. Formoltitration gave, however, the right value, 50 %, and then it turned out that pure asparagine (kindly supplied from the Carlsberg Laboratory) gives also too low amino-N values in the colorimetical method; the apparent amino-N was found to be 37.3 % of the total N.

A solution of the asparagine isolated was subjected to carbon adsorption (USSING 1945 b). The amino-N decreased 20 % per carbon treatment, exactly what was found for pure asparagine.

(From the same blood filtrate a small crop of tyrosine was isolated and identified by the crystal form, low solubility in water and an intense Millon reaction.)

As mentioned above the presence of an amide in the monamino acid fraction was first indicated in Oryetes and it was later reaffirmed (compare USSING 1945 a). As soon as Oryetes larvae became again available,  $\frac{24}{4}$ , an attempt was made to isolate asparagine from these animals. The blood from 25 larvae, 27 ml, was used and the monamino acid fraction prepared as usual (55 ml). The solution was neutralized to litmus and mercuric acetate and sodium hydroxide were added by drops until no more precipitate would form. The precipitate after washing was suspended in water and decomposed with  $H_2S$ . The solution was concentrated practically to dryness, but no asparagine crystallized. The only crystals formed were some fine needles at first suspected to be tyrosine. The sample was stored for later examination.

During the preparation of the monamino acid fraction used it

was noticed, however, that the addition of hot  $\text{Ba}(\text{OH})_2$  solution to remove phosphotungstic acid produced a strong smell of ammonia although the blood does not contain this substance to a measurable extent. This means that a substance is present, which splits off ammonia in alkaline solution even at temperatures appreciably below 100°. It could hardly be asparagine because this substance is relatively stable and it was therefore suggested that the substance in question was glutamine. Whether glutamine or another instable substance it was evident that the method hitherto used was likely to destroy a considerable part of the substance. Treatment with hot alkali should be omitted and it was therefore decided in the next experiment to precipitate the supposed glutamine before the PW-precipitation. (Another possibility was to remove excess phosphotungstic acid by extraction with n-amylalcohol and ether; but it has been impossible to get n-amylalcohol during the war.) The most simple way would have been to precipitate amides with mercuric nitrate after removal of interfering substances with Pb-acetate (compare for instance VICKERY *et al.* 1935 a); but in this way a considerable amount of nitrate ion would be introduced which could not be removed again, and the filtrate from amide precipitation had to be reserved for other analyses (see below). Tests with asparagine and urea (glutamine was not accessible) showed that precipitation with Hg-acetate was not quantitative in dilute solutions as is the precipitation with Hg-nitrate. The precipitation of asparagine with Hg-acetate was, however, much improved when 3 volumes alcohol were added, while on the other hand this treatment did not precipitate most monaminomonocarbonic acids.

The procedure adopted was therefore as follows: Blood-filtrate was prepared as usual from 26.9 g's of Oryctes-blood (Oryctes  $\frac{2}{5}$ ). After concentration in vacuo to 50 ml, lead acetate was added until no more precipitate was produced. The solution which was first acid to Kongo-paper became neutral (due to precipitation of the sulphate and phosphate ions). After removal of the precipitate, mercuric acetate was added until no more precipitate would form. Then 3 volumes alcohol were added and the precipitate allowed to settle in the cold overnight. The precipitate was washed with 80 % alcohol, suspended in water and decomposed with  $\text{H}_2\text{S}$ .  $\text{H}_2\text{S}$  was removed by concentration in vacuo. Some contaminating substance (histidine ?) was precipitated with a little phosphotungstic acid after addition of some drops of sulphuric acid. Most of the  $\text{H}_2\text{SO}_4$  was removed with  $\text{Ba}(\text{OH})_2$ . The last trace of  $\text{H}_2\text{SO}_4$  and the excess phosphotungstic acid was removed with lead acetate. Lead was precipitated with  $\text{H}_2\text{S}$ . The solution was con-

centrated in vacuo to 2 ml. It was then transferred to a small porcelain dish and a little ammonia was added to neutralize the acetic acid. By gentle heating on a waterbath the solution was concentrated in a strong air current to speed up the evaporation without undue heating (glutamine decomposes rapidly even at neutral reaction, when the temperature is above 60°). An oily remanence was left: about 5 volumes of alcohol were added and after vigorous stirring for several minutes the oily remanence would crystallize in very small needles. Tyrosine was only present in traces (Arnow's modification of the Millon reaction (1937)).

According to VICKERY *et al.* (1935 b) glutamine splits off all or nearly all its amino-N when it is heated to 100° for 2 hours at pH 6.5. Asparagine gives no ammonia under these conditions and even urea and allantoin which are relatively unstable are only partly split in two hours, so that heating for four hours will give much more ammonia than heating for two hours. These authors suggest that these facts may be used to differentiate between the amides mentioned.

The following experiment was made: 13.93 mg of the powder, supposed to contain glutamine was dissolved in 7 ml water. One ml samples were mixed with 1 ml each of phosphate buffer, pH 6.5, in test tubes which were sealed off and heated in boiling water for two and four hours. Then the tubes were placed in an ice-bath and the water condensed in the upper end which may contain ammonia driven down by gentle heating with a free flame. The ampoules were opened and the contents transferred to the distillation flask of a distillation apparatus of the Parnas type together with the washings, 2 ml. 2 ml 1% Na<sub>2</sub>CO<sub>3</sub> were added and the ammonia distilled over in 1 ml  $\frac{n}{100}$  H<sub>2</sub>SO<sub>4</sub>.

Baektitration with  $\frac{n}{20}$  NaOH. Indicator: Mixture of methylred and methylene blue. Results are shown in table III.

Table III.

ml NaOH used

1 ml $\frac{n}{100}$ H <sub>2</sub> SO <sub>4</sub> .....	187
1 ml sample + 1 ml buffer, not heated .....	{ 98.8 96.2
* * * + * * * , heated for 2 hours .....	{ 26.1 24.0
* * * + * * * , heated for 4 hours .....	{ 19.6 16.6

In two hours thus the equivalent in NH<sub>3</sub> of 72.3  $\mu$ l NaOH was found, whereas in four hours the titration gave 79.2  $\mu$ l. This

means that more than 90 % of the ammonia was released within 2 hours.

In order to purify the supposed glutamine, the remaining sample was boiled with an excess of freshly prepared Cu(OH)<sub>2</sub>, filtered hot and concentrated to a small volume. The next day the syrup had crystallized. The crystals were digested twice with 0.3 ml water and dried: 35.4 mg

15.0 mg copper salt was dissolved in water and decomposed with H<sub>2</sub>S. After removal of H<sub>2</sub>S the solution was made up to 10 ml and the glutamine content was determined as above with the exception that 1 ml 5 % borax in 0.5 n NaOH was used instead of Na<sub>2</sub>CO<sub>3</sub> for alkalizing. The results were (table IV):

Table IV.

	$\mu\text{l}$ NaOH used
1 ml $\frac{n}{100}$ H <sub>2</sub> SO <sub>4</sub> .....	187
1 ml sample + buffer, not heated .....	157.6
	156.7
* * * + * , heated for 2 hours .....	{ 90.0 88.5
* * * + * , heated for 4 hours .....	{ 85.4 86.5

In two and four hours respectively the NH<sub>3</sub>-equivalent in NaOH of 67.9  $\mu\text{l}$  and 72.2  $\mu\text{l}$  were released. This means that about 94% decomposition takes place within 2 hours. It is seen that the substance contains still some ammonia, the equivalent of 20  $\mu\text{l}$  NaOH per ml solution, and correspondingly the content of amide-N is somewhat low, 5.4 % of the copper salt instead of 6.7 % as calculated for glutaminecopper. Further recrystallization was abandoned on account of the small amount of substance left.

The substance isolated by Hg-acetate precipitation from Oryctes blood, monamino acid fraction (see above), was now dissolved in water and aliquots heated at pH 6.5. It proved that this sample also contained glutamine: the ammonia set free in 2 hours was 98 % of that formed in 4 hours.

Although glutamine was not isolated in the pure state, its presence in Oryctes blood must be regarded as demonstrated. Its identification rests on the following facts: The blood contains an amide, which is not precipitated by phosphotungstic acid, lead acetate or baryumhydroxide and alcohol. It forms a copper salt, which is but little soluble in water (difference from urca, allantoinic a. o.) and it splits off nearly all amide-N within 2 hours

when heated to 100° at pH 6.5 (difference from asparagine, urea and allantoine). It is insoluble in alcohol and crystallizes in fine needles. Even the tendency to form supersaturated solutions is characteristic (compare VICKERY *et al.*, (1935 a)).

The next question was if glutamine is also found in the blood of Melolontha.

Blood was taken from 25 larvae ( $\frac{2}{3}$ ) and the protein- and trichloroacetic acid free extract was prepared as usual. The solution was acidified with sulfuric acid and bases and peptides precipitated with phosphotungstic acid.  $H_2SO_4$  and phosphotungstic acid were removed with lead acetate and lead with  $H_2S$ . Acetic acid was removed after concentration by extraction with ether, 4 ml solution, corresponding to 1.11 g blood were mixed with 4 ml buffer (pH 6.5). Using a glass electrode pH was adjusted exactly to 6.5 with  $\frac{n}{20} NaOH$ . 2 ml samples of this solution each corresponding to 0.241 g blood were heated as above for 2 hours. Ammonia was formed corresponding to 0.188 mg N per blood, or to 196 mg % glutamine.

The rest of the blood filtrate was freed from a trace of phosphotungstic acid with  $Ba(OH)_2$  and  $Ba^{++}$  removed with  $H_2SO_4$ . The resulting solution was adjusted to pH 6.5. 6 two ml's samples were mixed with 1 ml buffer (pH 6.5) each and two 2 ml samples were mixed with 1 ml 10 %  $H_2SO_4$ . The further treatment and the results are seen from table V; each sample corresponds to 0.41 g blood.

Table V.

	$\mu l$ NaOH used
1 ml $\frac{n}{100} H_2SO_4$ .....	187
pH 6.5; not heated .....	168.5
	168.5
pH 6.5; 2 hours' heating .....	80.1
	83.8
pH 6.5; 4 hours' heating .....	78.0
	79.2
Strongly acid; 3 hours' heating .....	68.1
	66.0
Blank on 1 ml 10 % sulphuric acid .....	180

The samples containing 10 %  $H_2SO_4$  were made alkaline with a mixture of the equivalent amount of NaOH and the usual amount of borax-buffer. The ammonia set free in 2 hours at pH 6.5 is 97 % of that formed in 4 hours. What is of special interest in this case is, that when correction is made for the small  $NH_3$  content of the sulphuric acid, practically the same amount of ammonia is set free at pH 6.5 and under the much more strenuous hydrolysis with sulphuric acid. But this means that these

larvae contain glutamine as the only amide. No or practically no asparagine is present in this case.

A similar experiment on Oryctes blood filtrate gave an amide-N content of 0.142 mg/g blood, corresponding to 150 mg% glutamine.

### 6. The behaviour of blood amino-N on carbon-adsorption.

In a previous paper (USSING 1945 b) the decrease in amino-N on repeated adsorptions on carbon under standard conditions was used to characterize certain amino acids. This method may be used to find out if an amino acid mixture contains to any extent glycine, alanine or other little adsorbable amino acids.

The procedure is described in detail in the paper mentioned above. Suffice it to say that in each treatment 30 mg carbon per ml solution was used so that the adsorption was only a function of the nature of the amino acids in question and of the concentration. It was further found that in the very dilute solutions used (1—5 mg % amino-N) the adsorption was approximately proportional to the concentration, or in other words the relative adsorption was independent of the concentration.

Table VI.

	% adsorption per carbon treatment
Glycine .....	1.5
Serine .....	5
Alanine .....	6
Asparagine .....	20
Glutamic acid .....	20
Lysine .....	24
Valine .....	27
Leucine .....	60
Histidine .....	84
(Glucose .....	70)

Table VI shows the approximate decrease per carbon treatment of some amino acids; glucose in similar concentration is also included for comparison, pH in all cases 7.

Many such adsorption analyses were made on blood extracts from Oryctes and Melolontha, on fractions as well as on unfractionated blood filtrates. In all cases it was found that the monamino acid fraction contained a considerable proportion of little adsorbable amino-N. Examples are shown in fig. I. Apart from adsorption curves for monamino acid fractions from Oryctes and Melolontha, the figure shows the curve for unfractionated blood

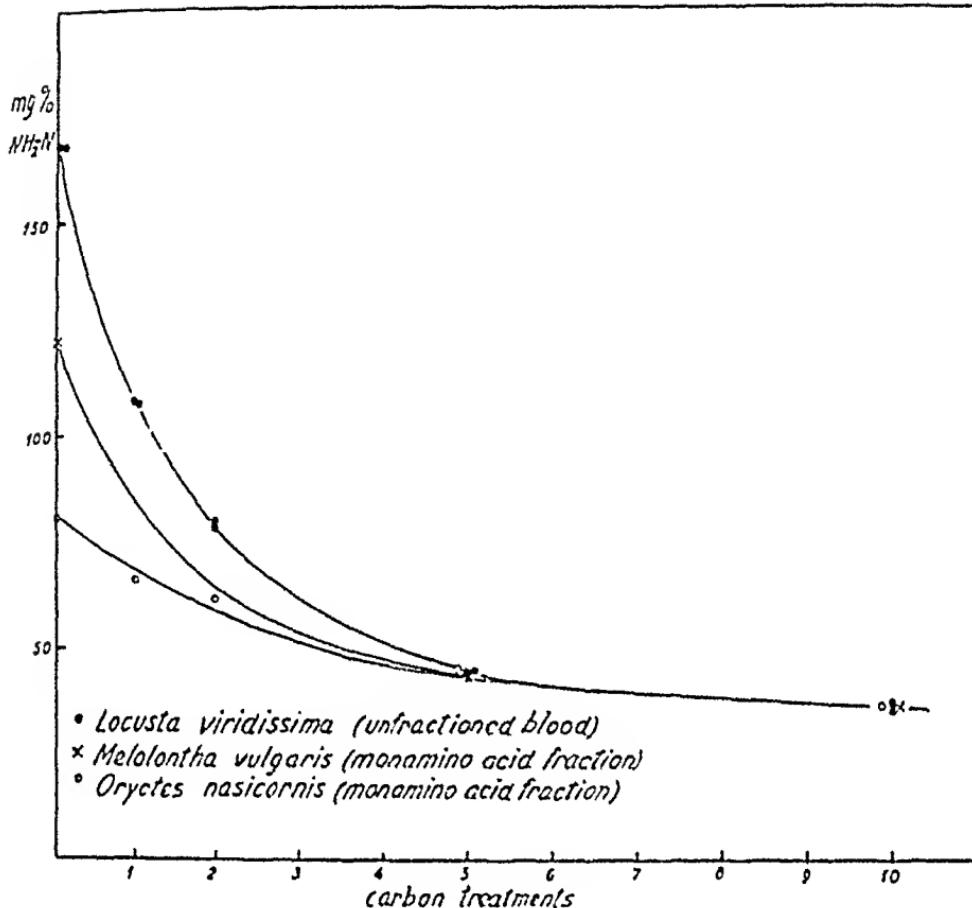


Fig. 1.

filtrate from imagines of the grasshopper *Locusta viridissima*. Incidentally the curves happen to have the endpoint in common and this allows an easy comparison between them. All adsorptions are performed on very dilute solutions, but the results are recalculated to the original concentration in the blood. It is seen that after 5 carbon treatments which remove most amino acids, the curves for the three species are nearly identical. The decrease per carbon treatment is between 4 and 5 %.

The presence in the blood of *Oryctes*, *Melolontha* and *Locusta* of one or more of the little adsorbable amino acids in a fairly high concentration is thus ascertained.

The decrease, 4—6 % per carbon treatment shows, however, that the amino-N left after 5 treatments does not come from pure glycine (decrease 1.5 %). The substances, which may come

into consideration, are apparently glycine, serine and alanine, moreover are proline and hydroxyproline known to be very little adsorbed by carbon (TISELIUS 1941). Attempts were therefore made to identify one or more of these amino acids.

*Serine.* According to RAPORT (1937) this amino acid may be estimated by being transferred by deamination to glyceric acid, which gives a specific reaction with naphthoresoreine. Dicarbonic acids and hexonbases should be removed. Sugars too interfere.

The blood filtrates (monamino acid fraction) gave no Fehling reaction; but with  $\alpha$ -naphthole and sulphuric acid they gave a violet colour. It proved, however, that three treatments with carbon (see above) removed the sugar as estimated by the  $\alpha$ -naphthole reaction, whereas serine, if present, should only be reduced some 14 %.

Several determinations were made both on Oryctes blood and on the blood of the larvae of the beetle Rhagium mordax; but in no case the blue colour characteristic for serine was obtained. A small content of serine would, however, escape detection because a brown colour always occurred, when insect blood was examined. A similar colour was also produced by some amino acids as for instance glycine and alanine.

It thus seems that serine, if present at all, is quantitatively unimportant in Oryctes blood.

*Alanine.* FURTH, SCHOLL and HERMANN (1932) determined alanine in protein hydrolysates by converting it into lactic acid by deamination. The lactic acid was determined according to FRIEDMANN and KENDALL (1929). Dicarbonic acids and the bases, which may also give rise to some lactic acid should be removed before the deamination. Since this method was worked out threonine has been recognized as a constituent of many proteins, and this amino acid will probably also form laetic acid under the above conditions.

As no Oryctes larvae were available at that time, determinations were only made on blood from Melolontha. As an example the following experiment may be cited:

A monamino acid fraction was prepared as usual. 25 ml of this fraction, corresponding to 12 g's of blood were treated twice with carbon (see above) which removes impurities without adsorbing much alanine. From the resulting solution 10 ml were used for direct laetic acid determination and 10 ml deaminized and then used for laetic acid determination, thus giving the sum of preformed laetic acid and laetic acid formed from alanine (and threonine)

10 ml not deaminized used .....	1.05 ml	$\frac{n}{100}$	J <sub>2</sub>
10 ml " " " .....	2.50 ml	"	"
lactic acid from alanine thus used .....	1.45 ml	"	"

This corresponds to about 0.93 mg alanine or 0.2 mg alanine per g blood.

Even this low value is, however, too high because, as was later found the monamino acid fraction contains glutamine and sometimes asparagine, both of which will give rise to lactic acid on deamination and oxydation. It thus seems that alanine (and threonine) are only present in Melolontha blood in quite small amounts, if at all.

*Hydroxyproline.* Glycine, proline and hydroxyproline form characteristic picrates. Especially the picrates of glycine and proline are little soluble and should be suitable for the isolation of those amino acids. Therefore the filtrate from the glutamine precipitation on Oryctes blood (Oryctes 2%, see above) was used for the attempt to identify as the picrates one or more of the amino acids mentioned.

The aleoholic filtrate was concentrated in vacuo to remove alcohol. Hg<sup>++</sup> was removed with H<sub>2</sub>S and H<sub>2</sub>S by distillation. Bases were precipitated with phosphotungstic acid after addition of some drops of sulfuric acid. The filtrate was freed from phosphotungstic and sulfuric acid with an excess of Ba(OH)<sub>2</sub> and the Ba<sup>++</sup> was exactly precipitated with H<sub>2</sub>SO<sub>4</sub>. Acetic acid which originated from the Pb- and Hg-acetates was extracted with ether. After removal of ether by vacuum distillation the solution was made up to 100 ml and shaken with 6 g carbon (SCHERING's Carbo ossium pro analysi). After filtration the carbon treatment was repeated with 5 g's of carbon. The filtrate from the second treatment now neutral to litmus was concentrated to dryness in a porcelain dish. The residue was extracted with a minimum of hot water and the resulting solution (about 200  $\mu$ l) was mixed with 5 volumes of alcohol. A precipitate formed which proved to consist of inorganic sulfates, whereas all amino-N remained in the alcoholic solution. This solution was concentrated to a syrup (50  $\mu$ l), but only a few cubic crystals of alkali chloride appeared.

The syrup was dissolved in two ml water and from this solution aliquotes were taken for amino-N determinations according to FOLIN (modified, see USSING, I. c.) and VAN SLYKE.

FOLIN: 4.80 mg N in whole sample; VAN SLYKE: 1.28 mg N in whole sample.

The difference 3.52 mg N must originate from amino acids which are determined with the colorimetric method and not with the van Slyke method. From the known amino acids this is only the case with proline and hydroxyproline and it is thus strongly indicated that one of these amino acids — or both — are present in Oryetes blood in considerable amounts.

GRASSMANN (1935) has described a characteristic reaction for these two amino acids. When they are heated in watery solution with a little isatin a blue colour is produced. The coloured substance is eagerly adsorbed on fibers of cotton or acetate-cellulose.

A drop of the solution which was being examined was boiled with a little isatin for two minutes with 1 ml phosphate buffer (pH 7) and the solution soon turned green. A tuft of cotton wool was placed in the solution, boiled and washed with hot water; it had turned intensely blue.

1.4 ml still remained of the "little adsorbable amino acid fraction" (Oryetes  $\frac{2}{5}$ ). It was concentrated to 0.5 ml in a small glass tube, using an air current to speed up evaporation. 40 mg picroic acid were added under stirring at 100°. On cooling crystallization began and the whole sample became a mass of long needle-like crystals. Under the microscope they appeared exactly like hydroxyproline-picroate with the characteristic tendency to form "brushes" (see CROSBY and KIRK 1935). The substance was rather soluble in water in contrast to proline-picroate. The crystals were sucked off on a micro glass filter, dried and washed with ether; crop: 30 mg. 26.8 mg picroate were dissolved in 8 ml water and after addition of 3 drops of 10 %  $H_2SO_4$  the picroic acid was extracted with ether. After removal of  $H_2SO_4$  and ether aliquots of the solution were oxidized with hypochlorite and subjected to a vapour distillation all according to WALDSCHMIDT-LEITZ *et al.* (1934). Under these conditions from all known amino acids only hydroxyproline gives pyrrole to be shown by a violet colour which develops when dimethylaminobenzaldehyde and hydrochloric acid is added to the distillate. The reaction was positive for the picroate-fraction of Oryetes blood and so the presence of hydroxyproline was finally demonstrated.

Untreated blood filtrate from Oryetes was also tested for hydroxyproline according to WALDSCHMIDT-LEITZ and with strongly positive result but due to lack of a hydroxyproline standard the test could not be used for quantitative determinations.

Whether proline is also present has yet to be found out; but

the high solubility of the picrate and the crystal form speaks against the assumption that more than a trace of this amino acid is present. In another experiment in which picrates were isolated from *Oryctes* blood in essentially the same way as that just described, the picrates were recrystallized from hot water, with the result, however, that only the picrate of an inorganic ion, presumably potassium was left. Thus no insoluble or little soluble amino acid picrates like those from proline or glycine were found.

The fact that the *Oryctes* blood filtrate even after carbon adsorption gives some amino-N in the van Slyke apparatus indicates that beyond hydroproline other little adsorbable amino acids are present in minor amounts. Due to lack of material similar analyses have not been made on *Melolontha* blood.

#### 7. The concentration of certain amino acids in blood and intestinal contents of *Melolontha*.

Blood extract from 17 *Melolontha* larvae (4.5 g blood) was prepared in the usual way. The blood protein was filtered off on a weighed ash-free filter, washed with water and dried to constant weight at 105°: 0.160 g or 3.55 %.

Contents from the mid-gut was collected in the following way: A sagittal incision was made in the skin in the midline of the back of the larvae. As a rule the midgut would then protrude in a hernialike fashion. Blood adhering to the gut was removed with filter paper and the contents of the gut could be collected from an incision in the gut wall in to a weighed vessel, containing 2 ml of 20 % trichloroacetic acid.

2.24 g gut-content was taken and then the solution was made up to 22.1 ml and filtered. The insoluble residue on the filter was washed and dried: 0.283 g or 12.65 %. The filtrate freed from trichloroacetic acid in the usual way was quite dark from some huminlike substance.

The colour was, however, practically removed by precipitation with bariumhydroxide and the remaining colour was adsorbed on the  $\text{BaSO}_4$ , when the  $\text{Ba}^{++}$  was removed with  $\text{H}_2\text{SO}_4$ .

For the sake of comparison the blood filtrate was likewise treated with  $\text{Ba}(\text{OH})_2$  and then freed from  $\text{Ba}^{++}$  again.

On the filtrates thus prepared analyses were made for total amino-N, histidine (according to RACKER (1940)), tryptophane (according to WINKER (1934)), tyrosine (according to ARNOW l. c.) and leucine + valine (USSING, 1943). The results of these analyses are presented in table VII, column I and II. In column III and IV the results are calculated on the basis that the dry substance, insoluble in trichloroacetic acid is not acting as solvent for the amino acids. For the blood an estimate is given for leucine

Table VII.

	Blood mg %	Intestinal contents, mg %	Corrected for dry substance		
			Blood mg %	Intestinal contents, mg %	Intestinal contents Blood
Amino-N .....	342	67.5	354	77.3	0.22
Histidine .....	300	c. 100	311	c. 112	c. 0.36
Tryptophan .....	12.5	0	13	0	0
Tyrosine .....	106	37	110	42	0.38
Leucine + valine (as leucine) .....	226	66	234	76	0.33
Leucine .....	131	—	—	—	—
Valine .....	88	—	—	—	—
Dicarbonic acid N ...	31.2	—	32.4	—	—

and valine separately; the procedure used is described in the next section.

From the table it is seen that all amino acids examined are present in higher concentration in the blood than in the gut. In another case ( $\frac{27}{10}$ ) amino-N determinations were also made on intestinal contents. The concentration was 48.8 mg% against 453 mg% in the blood. We shall return to this point later.

### 8. Differential estimation of leucine and valine.

In their original method FROMAGEOT and HEITZ (1939) used the different amounts of acetone formed on short and prolonged chromic acid oxydation from deaminized leucine and valine for the differential estimation of the two amino acids. BLOCK (1940) used oxidation with two different oxydants (chromic acid and permanganate) for the same purpose.

When very small amounts of the two amino acids are to be determined, these methods present serious difficulties, and it was therefore attempted to modify the method worked out by the author (USSING 1943) for the determination of the sum of leucine and valine so as to permit differential determinations.

As shown in table VI valine is much less adsorbed by carbon than is leucine, and this fact is made use of in the estimation of the two amino acids.

The best differentiation proved to result when 60 mg carbon was used per ml solution.

The estimation is made in the following way: From the solution to be examined a sample containing 0.5—1 mg leucine + valine

is taken for the direct determination of the sum of these amino acids. Another sample containing 2—4 mg leucine + valine is neutralized to lithmus and diluted to 10 ml. 600 mg carbon is added and the mixture shaken thoroughly. The carbon is filtered off and the filtrate is measured with an accuracy of 0.05 ml, transferred to a test tube, deaminized, concentrated and oxidized in sealed ampoules upon which the distillation of acetone and the colour development is performed in Conway units as usual.

For the calculation the following empirically found facts have been used: 1) Equimolar amounts of leucine and valine give equal amounts of acetone on direct determination. 2) Leucine decreases to 13.5 % on carbon treatment whereas 3) valine decreases to 41.5 %.

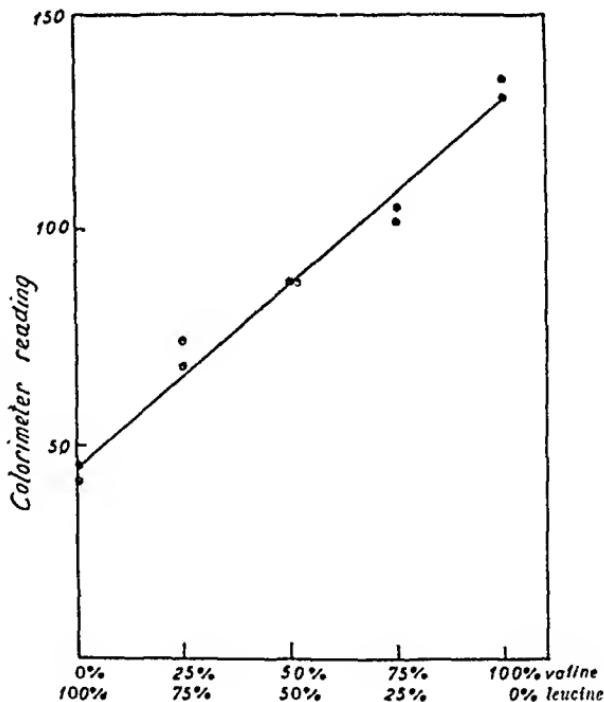


Fig. 2.

Fig. II shows the influence of adsorption on known leucine-valine mixtures. The ordinates (in arbitrary units) are corrected for blank and calculated under the assumption that the molar equivalent of 0.5 mg leucine is analyzed each time. Without adsorption 0.5 mg lucine or the equivalent amount of valine would give 320 arbitrary photometer units.

The error on the valine or respectively the leucine values may sometimes exceed 10 % but still this method allows an approximate determination of the amino acids in question in extremely small samples.

### 9. Variations in total amino-N in the blood filtrates.

Considerable variations were found in the non-protein amino-N of the blood filtrates. Table VIII shows examples of these variations together with remarks about the conditions under which the samples were taken.

Table VIII.

	mg % NH <sub>2</sub> -N
Animals directly from habitat ( <i>Oryctes</i> $\frac{22}{15}$ ) .....	316
" " " " " (" " $\frac{21}{15}$ ) .....	219
Animals kept at room tp. (20—22°) for 11 days ( <i>Oryctes</i> $\frac{23}{11}$ ) .....	284
Animals directly from habitat ( <i>Melolontha</i> $\frac{23}{10}$ ) .....	375
" " " " " (" " $\frac{22}{10}$ ) .....	453
Animals kept at room tp. for 10 days ( <i>Melolontha</i> $\frac{19}{10}$ ) .....	342
" " " " " (" " $\frac{25}{10}$ ) .....	280

## Discussion.

In the two species studied, *Oryctes* and *Melolontha*, the non-protein amino-N of the blood has been found to originate from three main groups of substances: Low peptides (di- and tripeptides), amides (glutamine and sometimes asparagine) and real amino acids. Nearly all nitrogen can be accounted for as belonging to the groups of substances mentioned.

In *Melolontha* blood the presence of the following substances has been established: The bases lysine, arginine and histidine, the monoamino acids tyrosine, leucine, valine and tryptophane and the amides glutamine and asparagine. From the shape of the carbon adsorption curve (see fig. I) the presence of a considerable amount of hydroxyproline is indicated. Moreover a small amount of peptide and possibly some diearbonic amino acid is found. Of these substances lysine, arginine and leucine were isolated by ACKERMANN (1920, 1921) in extracts from whole *Melolontha* larvae, but as the high amino-N content of insect blood was not known at that time, the amino acids mentioned were apparently assumed to be tissue extractives. ACKERMANN did not isolate tyrosine. Probably it had been destroyed by enzymes in the

tissue mince. Perhaps the p-hydroxyphenylethylamine isolated by ACKERMANN had been formed in this way.

In Oryctes blood conditions are quite similar as far as the analyses go. A quantitative difference is found in the monoamino acid fraction, which is in this species apparently dominated by hydroxyproline and glutamine. The moderate decrease on carbon adsorption (see fig. I) shows that the concentration of the strongly adsorbed amino acids, for instance leucine and tyrosine must be low and this fits in with the fact that no tyrosine could be isolated from the Hg-precipitates from Oryctes in contrast to that from Melolontha.

It cannot be ruled out, however, that seasonal changes may have something to do with the differences found. In this connection the curious fact that asparagine was found in Melolontha in the autumn but not in the spring may be mentioned. The difference may be due to changes in metabolism; but another possibility is that asparagine may be taken up by the animals from the plants on which they feed and stored for some time in the blood before it is split or excreted.

The glutamine, which is found in both species, may of course originate from plant-food too, but its presence might just as well have some connection with the syntheses of uric acid. As shown by ÖRSTRÖM, ÖRSTRÖM and KREBS (1939) the uric acid formation in liver slices from birds is speeded up on glutamine addition.

The question put in the introduction: are insects able to transfer amino acids from a low concentration in the gut to a higher concentration in the blood, can now be answered in the affirmative with some certainty. Not only the total amino-N, but also the individual amino acids analyzed for are much more concentrated in the blood than in the intestinal contents (see table VII), even when correction is made for the higher content of dry matter in the gut. As most of the osmotic pressure in insect blood is due to amino acids it seems likely that the blood and the contents of the gut are very far from isotonic. It is a type of absorption which reminds of the reabsorption of substances in the tubules of the kidney of vertebrates. It is possible that the conditions found are peculiar for the very curious digestive system of the lamellicorn beetles. Studies on other types are needed.

The total concentration of amino-N in the blood is subject to considerable fluctuation in both species studied (see table VIII). Nothing definite can be said about the reasons for these fluctua-

tions, but as a working hypothesis it is supposed, that temperature has something to do with the phenomenon. Taking *Oryctes* first, it is seen that the concentration is lowest when the outer temperature is highest. The reason why the animals used  $^{22}/_{11}$  had been kept at room temperature was that due to high viscosity practically no blood could be obtained 10 days before when the animals had been kept at  $10^{\circ}$  for some time.

In the case of *Melolontha* special interest should be paid to the analyses for the dates  $^{23}/_{10}$  and  $^{27}/_{10}$ . These two days and again the  $^{31}/_{10}$  animals were collected on the same locality. In the course of that week the weather became cold and the  $^{31}/_{10}$  only 2 animals were found. The others had left the upper strata of the earth and dug down to the wintering level. In this week the amino-N increased from 375 mg % to 453 mg % and the total non-protein N from 447 to 643 mg %. An excretion of water (and possibly some amino-N) would be the simplest explanation on this phenomenon. That insects concentrate their blood under diapause is well established (compare MELLANBY, 1938), but possibly a more general dependence of outer temperature exists.

Although a high amino-N content is common to all insects studied there are apparently considerable differences as to the relative concentration of the individual amino compounds between the different insect species. FLORKIN and DUCHATEAU (l. c.) were unable to find for instance arginine and tryptophane in adult *Dytiscus*, whereas both amino acids were found in *Melolontha* larvae. Perhaps the reason is that the haemolymph of the larvae is acting as a deposit for substances, which are used for protein synthesis during the metamorphosis (compare HELLER, 1932). Arginine moreover may leave the blood to be stored in the muscles as phosphoarginine.

The adsorption curve for unfractionated blood from *Locusta viridissima* (fig. I) shows clearly that no single amino compound dominates the picture in adult insects. At least two and possibly more substances are of importance. The slope of the curve between the 5' and the 10' carbon treatment justifies the search for hydroxyproline in this species too. Much more work has to be done, however, before a clear picture of the amino acid composition of insect blood has been obtained.

The author wishes to express his gratitude to Miss GIERLOFF who has performed much of the analytical work.

### Summary.

1. The haemolymph of the larvae of the beetle *Melolontha vulgaris* was found to contain between 280 and 450 mg % non-protein amino nitrogen; in haemolymph from the related *Oryctes nasicornis* between 219 and 316 mg % was found.
2. This amino nitrogen originates from three main sources, namely a) real amino acids, b) low peptides (di- and tripeptides) and c) amides (glutamine and asparagine).
3. In *Melolontha* blood the presence of the following substances was established: lysine, arginine, histidine, tyrosin, leucine, valine, tryptophane and the amides asparagine (in some cases) and glutamine; the presence of hydroxyproline in an appreciable amount was indicated. Moreover a small amount of peptides and some dicarboxylic amino acid or acids was present.
4. In *Oryctes* blood hydroxyproline was identified; this substance and the basic amino acids play the most important part among the real amino acids. Low peptides are present in considerable amounts. Glutamine seems always to be present, whereas asparagine has not been met with.
5. The non-protein amino-nitrogen in the content of the mid-gut of *Melolontha* has been examined. It was found that the concentration of all amino acids analyzed for was much lower in the gut than in the haemolymph.
6. The variations in the amino-N concentration of the haemolymph is briefly discussed.

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## The Influence of Anoxia on the Gastric HCl-secretion.

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The gastric HCl-secretion varies sensitively owing to many different reasons. The classical experiments of PAVLOV, concerning conditional reflexes, give light to the part that the nervous system and physical actions play as regulators of the secretion, but the humoral agents regulate it also; thus gastrin, the hormone secreted in the pyloric part of the stomach, acts through blood as an irritant of the HCl-secretion. Some other alterations in the composition of blood have also influence upon the secretory function of the stomach. It has also been ascertained, that the HCl-secretion decreases, when the organism becomes exposed to oxygen deficiency and alterations caused by it. BAYEUX (1910) observed a noticeable decrease in the quantity of secretion and a smaller one in the total acidity on a dog, when given a meal of meat in the height of 4,360 m. Similar results have, using dogs, later on DELRUE (1934), SLEETH and VAN LIERE (1936) as well as PICKETT and VAN LIERE (1941) attained. WARREN (1937) and HARTMANN, HEPP and LUFT (1941) have also observed, that the HCl-secretion produced by an alcohol test-meal given to the members of some mountain expeditions decreased already in the height of 4,000 m. HARTMANN, HEPP and LUFT observed besides, that the duration of the HCl-secretion decreased, when the height increased. — HELLEBRANDT, BROGDON and HOOPES (1935) on the other hand, when investigating the influence of a short duration of anoxia, came to a so far differing result, that "... acute anoxemia of the pre-coma type has relatively little inhibiting effect upon the secre-

tion of hydrochloric acid by the normal human stomach . . .", even though an evident influence is noticeable in their experiments too.

Both WARREN and HARTMANN, HEPP and LUFT consider the alkalosis caused by anoxia as the reason of the decrease of the HCl-secretion; in this case the HCl-secretion implies an extra transfer of acid ions from blood, already alkaline, and the increasing of alkalosis. It is likewise known, that a hyperventilation, voluntarily brought about, causes a great decrease in the HCl-secretion of the stomach. (DELHOUGNE, 1927; BROWNE and VINEBERG, 1932).

DELRIE in his experiments, which he performed in the height of 3,640 m, using histamine as the irritant of HCl-secretion, came to such results, that, when the dog becomes acclimatized to anoxia, the HCl-secretion, at first decreasing, in 2—3 hours regains its earlier level; the return of the HCl-secretion is accelerated by intense physical work. He does not think, however, that the reduction of HCl-secretion is caused by the immediate rise of the pH of the blood, because  $\text{NaHCO}_3$ , given intravenously, does not correspondingly decrease the HCl-secretion in spite of the rise of the pH of blood.

While JALAVISTO and the authors were investigating the HCl-secretion on decompensated heart patients,<sup>1</sup> there arose the question, whether the oxygen deficiency as such — irrespective of simultaneous variations in the ion-equilibrium — has a decreasing effect upon the HCl-secretion. VAN LIERE (1941) presents as his opinion, that ". . . the gastro-intestinal tract has a low energy requirement for secretion, since it is capable of withstanding relatively severe grades of anoxia, before it is significantly affected . . .", but he remarks also, that the question has been as yet insufficiently investigated.

The main purpose of this investigation was to throw experimental light on the influence of anoxia on man, and the plan was following: the subjects were to be brought in a condition of oxygen deficiency — e.g. by lowering the barometric pressure — but the accompanying production of alkalosis was to be prevented. There are three different possibilities:

- (1) mechanical prevention of hyperventilation;
- (2) increasing the  $\text{CO}_2$ -content of the inspired air;
- (3) giving of "fixed" acids to the subject.

<sup>1</sup> As yet unpublished.

The first of the above mentioned possibilities is of course out of question, as the experiments are made on man; the increasing of CO<sub>2</sub> as such increases HCl-secretion (BAKALTSCHUK, 1928; MOSONYI, GÜNTHER and PETRÁNYI, 1935) and causes a maximal hyperventilation, which partly compensates even a serious oxygen deficiency of inspired air. Giving of "fixed" acids — and salts acting as acids in organism — on the other hand causes hyperventilation in a relatively smaller degree (DOUGLAS, GREENE and KERGIN, 1933) and does not as such influence the HCl-secretion, as MACLAGAN (1934) with NH<sub>4</sub>Cl and SCHIFFLERS (1936) with H<sub>3</sub>PO<sub>4</sub> have experimentally proved.

### Method.

The experiments were performed using three healthy medical students in the age of 19—27 years: K. H., T. L. and M. K. The test-meals were done in the morning about 2 hours after awakening, when the stomach was empty. After the stomach-tube had been swallowed, the stomach was emptied 4 times at the intervals of 10 minutes, after which 300 ccm 5% alcohol, dyed with methylene blue, was poured in as irritant. After that six samples of gastric juice were taken likewise every 10 minutes; as long as the blue colour was observable, 10 ccm was taken at a time, and when the samples had changed colourless, all the gastric content available, likewise the 6th time, regardless of the colour. After the 6th sample 0,01 mg of histamine pro kg body weight was injected subcutaneously, and 10 min. after that the stomach was emptied again. On these samples the free HCl was titrated with NaOH using Kongo red, and the total acidity using phenolphthalein as indicators; when the acidity of the sample was on the alkaline side of the change-point of Kongo red, it was titrated with HCl until the change-point. The results are reported in the usual way in ccm of 0.1 N alkali or acid pro 100 ccm gastric content. The sample quantities, thus attained, were measured, and the dilution of colour in the samples was determined using comparison series. In a part of the samples the pH also was measured (electrometrically, with glass electrode).

In order to prevent the production of alkalosis, NH<sub>4</sub>Cl was taken in use. It was given to the subjects per os on the day before, 0.15 g pro kg body weight, divided into three portions, which dosage seemed to reduce sufficiently the alkali reserve of the blood, so that no alkalosis developed during the experiments. This was made sure by determining the pH of the subjects' urine before the test-meal, during it and after it. According to BRASSFIELD and BEHRMANN (1941) the pH of the urine reacts liability to that pH-rise of blood which is a result of hyperventilation caused by O<sub>2</sub>-deficiency, and rises about 1 pH-degree.

The oxygen deficiency experiments were performed in a lowpressure chamber. The subjects swallowed the stomach-tube at normal baro-

metric pressure — mountain-disease causes nausea, which makes the swallowing in low pressure very unpleasant — after which the first sample was taken immediately, and the pressure in the chamber was brought down in a few minutes to the pressure desired in each experiment. The low pressure was kept unchanged during the whole experiment. — To avoid the possible disturbing effect of acclimatization the experiments were performed during many weeks with irregular intervals and in variable order; for the same reason it was controlled, that the haemoglobin percentage of the subjects' blood remained constant during the experiments.

Each subject went through the following experiments:

(1) test meal in normal barometric pressure, in which the oxygen pressure in inspired air is 149 mm, when the air is saturated in 37°C with water vapour;

(2) as above after giving NH<sub>4</sub>Cl;

(3) and (4) as (1) and (2), but in 462 mm barometric pressure, corresponding to the height of 4000 m, oxygen pressure being 87 mm;

(5) and (6) as (1) and (2), but in 378 mm barometric pressure, corresponding to the height of 5,500 m, oxygen pressure being 69 mm.

In the 87 mm O<sub>2</sub>-pressure all the subjects were more or less cyanosed and did not feel very well. In the 69 mm O<sub>2</sub>-pressure all of the subjects were greatly cyanosed and felt seriously ill. In both of the O<sub>2</sub>-pressures the subjective condition was better after NH<sub>4</sub>Cl-administration than without it. Noticeable differences between various subjects were not observed.

## Results.

During the experiment it was observed, that free hydrochloric acid was secreted in the stomach of all the subjects. The emptying of the stomach on the subject M. K. was, as concluded from the small amounts of secretion received and the rapid dilution of the dye, more rapid than that of the subjects K. H. and T. L. After the alcohol irritation the acidity values on M. K. in all the experiments reached their maximum during the experiment, but the values on both of the others continually rose until the interruption of the experiment. — The variations in total acidity, HCl and pH were parallel to such an extent, that it makes almost no difference which of them is followed.

The acidity values of the first sample have not any special importance in our experiments, because the subjects were brought to O<sub>2</sub>-deficiency only after taking the sample. The other acidity values with empty stomach before the irritant alternated relatively irregularly, so that no conclusions can be drawn from them about the possible influences of oxygen deficiency.

Table.

*The Effect of Anoxia on HCl-secretion.*

Without NH <sub>4</sub> Cl-administration			Subject	After NH <sub>4</sub> Cl-administration			
The maximal values		The values after histamine injection:		The maximal values		The values after histamine injection:	
After alcohol irritation: TA / HCl	Time in minutes after alcohol (TA/HCl)			O <sub>2</sub> -pressure mm	After alcohol irritation: TA / HCl		
33.2/28.7	60/60	41.5/28.5	K. H.	149	70.0/49.0	69/60	
17.5/10.9	40/30	32.0/18.0		87	23.5/ 9.2	69/60	
21.5/13.4	60/60	26.0/17.5		69	17.0/11.5	60/60	
			T. L.				
53.0/26.7	60/60	65.1/36.8		149	28.0/22.7	60/60	
39.5/12.2	60/50	37.5/25.0		87	14.6/ 6.0	60/50	
12.5/ 7.0	60/60	16.5/ 6.0		69	9.0/ 4.5	60/50	
			M. K.				
45.2/31.6	40/40	58.2/35.7		149	66.2/47.6	50/50	
54.5/19.3	40/40	46.5/18.0		87	82.2/39.3	40/30	
38.5/14.3	10/10	35.5/ 7.5		69	38.5/10.1	20/50	

The most important values, obtained in the experiments, are collected into the table on this page. It presents side by side the greatest values of total acidity and HCl without NH<sub>4</sub>Cl-administration and after it with alcohol as irritant, and the time that elapses before the highest values are attained after giving alcohol; when the maximum had not been passed during the experiment, it was marked 60 minutes. The third pair of columns presents total acidity and HCl-values 10 minutes after injecting histamine.

(1) When comparing the results of acidity values in the different O<sub>2</sub>-pressures after NH<sub>4</sub>Cl-administration and without it, it is noticed, that there are no regular and essential differences either after alcohol or after histamine irritation.

(2) When the greatest acidity values received after alcohol irritation are examined, it is noticed, that:

/ (a) in 87 mm O<sub>2</sub>-pressure there were no essential and regular changes; in a part of the experiments the values were higher than normally, in a part lower, in the majority, however, lower;

(b) the acidity values in 63 mm O<sub>2</sub>-pressure were regularly decreased in a considerable degree, compared both to normal values and to those attained in 87 mm O<sub>2</sub>-pressure. Free hydrochloric acid was to be found in all the subjects, however.

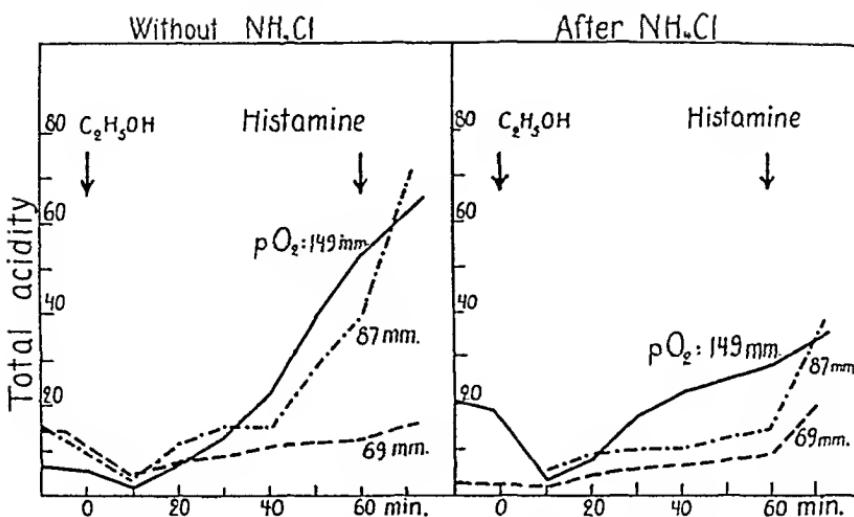


Fig. 1. The data of total acidity on the subject T. L. in different O<sub>2</sub>-pressures without and after NH<sub>4</sub>Cl-administration.

(3) The acidity values which were derived after histamine irritant show, that

(a) in 87 mm O<sub>2</sub>-pressure there were no regular variations; in a part of the experiments the acidity values had risen, in another decreased, when compared to normal values;

(b) the acidity values in 69 mm O<sub>2</sub>-pressure had as a rule decreased.

Because the experiments were interrupted, it is not known, how great the maximal acid secretion, produced by histamine, has been in the different experimental conditions. Accurate conclusions cannot be drawn from the fact, whether the oxygen deficiency in our experiments has decreased the HCl-secretion, caused by the injection of histamine. The subject M. K., whose reaction type was the fastest, had not very low acidity values after the histamine injection even in 69 mm O<sub>2</sub>-pressure. In all the experiments the histamine injection increased the acidity values from the level they had reached after alcohol irritation.

The curves of the subjects T. L. and M. K., presenting total acidity both without NH<sub>4</sub>Cl-administration and after it in different O<sub>2</sub>-pressures, give a clear idea of the influence of anoxia on HCl-secretion. (Figures 1 and 2.)

On the subject M. K. the maximum of HCl-secretion was attained earlier both in 87 mm and 69 mm O<sub>2</sub>-pressures in accord-

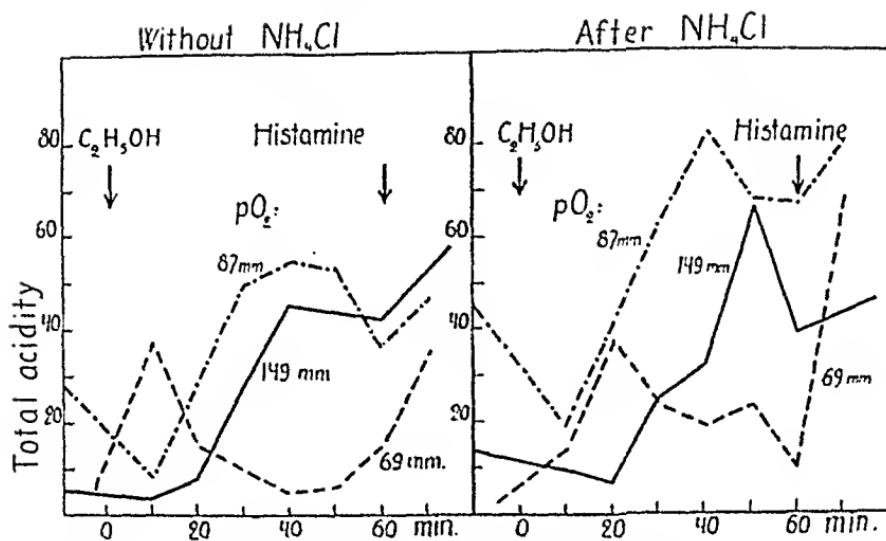


Fig. 2. The data of total acidity on the subject M. K. in different  $O_2$ -pressures without and after  $NH_4Cl$ -administration.

ance with the results of HAARTMANN, HEPP and LUFT. Evidently it does not mean only shortening of the secretion time, for, when inspecting the secretion curves, it is noticed, that the HCl-secretion occurred also more rapidly in normal conditions: the ascent of the curve is steeper. On the other subjects such shift of the maximum is not to be found in our experiments — very possibly due to the relative slowness of their HCl-secretion compared to the arrangement of our experiments. Even though the acidity values of the subjects K. H. and T. L. continually increased, it is very likely, that the reduction of the acidity values in their anoxia experiments is not a consequence of the retardation, but expressly of the decrease of the secretion. This is shown by the fact, that the slope of the graphic curves indicating HCl-values is already before histamine injection even on these subjects as a rule decreasing, most apparently in the anoxia experiments. It may be mentioned, that HELLEBRANDT, BROGDON and HOOPES in their experiments observed individual variations to some extent of the same type in the reactions of the subjects' HCl-secretion to  $O_2$ -deficiency.

The dilution of the dye may be regarded as an indirect measure of the quantity of the secrete in so far that, if the colour does not weaken, no secretion takes place; if, on the contrary, it becomes diluted, the quantities, both of the fluid secreted in the stomach, and that flowing from it, influence the rapidity of the dilution.

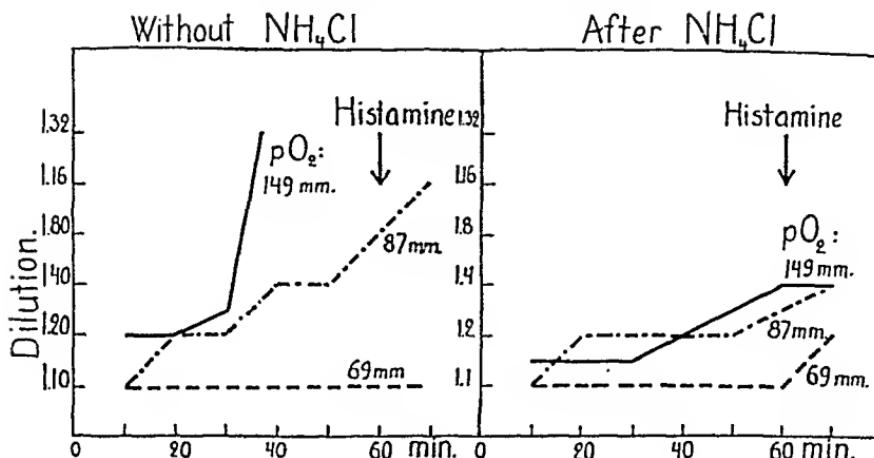


Fig. 3. The dilution of dye on the subject T. L. in different O<sub>2</sub>-pressures without and after NH<sub>4</sub>Cl-administration.

Depending on the abundance of the fluid, used as irritant, the amount taken as samples, has as such no significance to the rapidity of dilution, when, on the other hand, the influence of the quantity of fluid that passes the pylorus into the intestine is very essential. The dilution of the dye on each subject was in normal conditions and in 87 mm O<sub>2</sub>-pressure essentially just equally rapid. In 69 mm O<sub>2</sub>-pressure on the other hand the dilution on K. H. and T. L. was very much slower and the secretion correspondingly scantier. On the subject M. K. the dilution was on the contrary more rapid than in normal barometric pressure. This was certainly partly due to the faster secretion, but another reason may also be, that the stomach was emptied more rapidly than in normal conditions. According to VAN LIERE O<sub>2</sub>-deficiency — it is true — delays the emptying of the stomach, but he mentions, that rather great individual variations can be observed in this influence. On the whole the dilution curves of our subjects are parallel to the corresponding acidity value curves; even the dilution does not make any essential difference between the experiments without NH<sub>4</sub>Cl-administration and after it.

The graphic curves in fig. 3, showing the data on the subject T. L., presents clearly the influence of anoxia upon dilution speed of the dye.

The average of the urine-pH on the different subjects was about 6.67 in the experiments without NH<sub>4</sub>Cl-administration in the anoxia experiments, when the corresponding average after NH<sub>4</sub>Cl-

administration was only pH 5.14. In the experiments preceded by NH<sub>4</sub>Cl-administration the pH rose during anoxia in 87 mm O<sub>2</sub>-pressure about 0.58 pH-degrees, and in 69 mm O<sub>2</sub>-pressure it fell 0.11 pH-degrees; in the experiments without NH<sub>4</sub>Cl it rose correspondingly 1.15 and 0.53 pH-degrees. Consequently, NH<sub>4</sub>Cl-administration can be regarded as an effective antagonist to the production of alkalosis in our experiments.

### Discussion.

Earlier investigators have usually regarded the decreased HCl-secretion during anoxia as a result of the simultaneous variations in the ion-equilibrium of blood. It is known, that the disturbances of the ion-equilibrium of blood for some other reasons also lead to variations of the HCl-secretion. BAKALTSCHUK supposed, that the increasing of the HCl-secretion, produced by CO<sub>2</sub>-inspiration, is a compensative reaction, effected immediately by acidity, wherein the organism tries to get rid of an excess of acid even in this way; the decreasing of the HCl-secretion, caused by voluntary hyperventilation, was to be understood correspondingly (DELHOUgne). BROWNE and VINEBERG regarded the CO<sub>2</sub>-content of blood as decisive. The variations of the pH of blood or the CO<sub>2</sub>-content of it, produced in other ways, however, have not a similar effect on the HCl-secretion, which was explained by DELRUE and LACQUET (1934) so, that the variations in question of the HCl-secretion depend both on the contents of the Ca<sup>++</sup> and HCO<sub>3</sub><sup>-</sup>-ions in blood; only the increasing of one or the other or both of them would make the increasing of the HCl-secretion possible and vice versa. The concentrations of the Ca<sup>++</sup> and HCO<sub>3</sub><sup>-</sup>-ions again are correlated, but also connected to the concentrations of H<sup>+</sup>- and HPO<sub>4</sub><sup>--</sup>-ions in a way, which is nearest represented by the following equation, introduced by KUGELMASS and SHOHL (1924):

$$\frac{[\text{Ca}^{++}]^2 \times [\text{HCO}_3^-] \times [\text{HPO}_4^{--}]}{[\text{H}^+]} = \text{Constant.}$$

SCHIFFLERS (1936) and THIELE (1937) again are of the opinion, that Ca<sup>++</sup>-ions alone have importance to the HCl-secretion. — The Ca<sup>++</sup>-content of blood is decreased in alkalosis, caused by hyperventilation and increased in CO<sub>2</sub>-acidosis, so that even these theories would satisfactorily explain the influence of hyperventilation and CO<sub>2</sub> on the HCl-secretion. On the other hand, however,

an altogether opposite standpoint has been presented: GRANT (1941) declares, that the Ca-salts have an inhibiting influence on the HCl-secretion, produced both by nervous and chemical irritation.

It is known, that hyperventilation, caused by O<sub>2</sub>-deficiency, increases the pH of blood and decreases both the CO<sub>2</sub>- and Ca<sup>++</sup>-contents of it. These variations have apparently taken place even in our experiments. NH<sub>4</sub>Cl-administration, on the contrary, decreases the pH of blood and increases its Ca<sup>++</sup>-content, but it has a decreasing influence on the CO<sub>2</sub>-content of blood, just as O<sub>2</sub>-deficiency does. In our experiments NH<sub>4</sub>Cl cannot be noticed to have any essential decreasing or increasing effect on HCl-secretion; thus the decreasing of the HCl-secretion is not effected by any of these variations in the ion-equilibrium, but most likely by the direct influence of anoxia.<sup>1</sup>

It is not impossible, however, that the decreasing influence on the HCl-secretion of both the alkalosis, caused by voluntary hyperventilation, and anoxia is based — at least partly — on the same mechanism. According namely to CAMPBELL (1941) the O<sub>2</sub>-pressure in the tissues decreases, when the pH of blood increases, e. g. as a result of hyperventilation, occurred in normal barometric pressure. This decrease again is a consequence of the increasing affinity of haemoglobin to oxygen, when the pH rises, and the blood less readily transfers oxygen to the tissues. (The dissociation curve of O<sub>2</sub>-haemoglobin moves to the left.) Thus the influence of alkalosis could perhaps be founded on anoxia — and not vice versa.

According to MOSONYI, GÜNTHER and PETRÁNYI the increasing effect of CO<sub>2</sub> on the HCl-secretion is directed to the secreting

<sup>1</sup> After having finished the writing of this study, we received a publication — delayed because of the war — in which STÄMPFLI and ENDNER (1944) report investigations concerning HCl-secretion in the mountains. It is interesting, that they too have investigated the influence of previous NH<sub>4</sub>Cl-administration on the HCl-secretion. Still, they have observed, that NH<sub>4</sub>Cl prevents the decreasing of the HCl-secretion in the height. — The controversy between their results and ours is perhaps only apparent, for they have made their investigations in the height of only 3,457 m, in which height the O<sub>2</sub>-deficiency is compensated practically completely, at least at rest. The compensation happens through hyperventilation — and leads to a transitory alkalosis. In these conditions the beneficial effect of NH<sub>4</sub>Cl on gastric secretion is a double one. In increasing the hyperventilation it furthers the compensation of the O<sub>2</sub>-deficiency. By its acidifying action, on the other hand, it eliminates the alkalosis and with it the other possible cause of a decreased HCl-secretion. — In their investigation the test meals after NH<sub>4</sub>Cl-administration and without it are made on different subjects, which also makes the judgement of their results difficult as to our main question, for the individual variations in the HCl-secretion are rather great.

mucous membrane. DELRUE and LACQUET also are of the opinion that the  $\text{Ca}^{++}$ -ions have a direct rôle in the reactions of HCl-production. Our own experiments do not explain, what the effective mechanism of anoxia on HCl-secretion is, but it is quite probable, that the influence of anoxia also acts directly to the secretory cells.

The formation of hydrochloric acid from the NaCl is an endothermic reaction, that binds energy in plenty. The source of this energy is — so it must be supposed — one or other oxidative reaction in the secretory cells. It is very probable, that the HCl-secretion must diminish, as the supply of oxygen becomes difficult and the supposed energetic reaction is reduced — whatever the mutual amounts in blood of ions, participating in the HCl-formation itself, may be.

### Summary.

(1) The aim of this investigation is to unravel, whether anoxia as such has — independent of the accompanying variations of the ion-equilibrium of blood — any effect on the gastric HCl-secretion.

(2) For this purpose test-meals were given on three human subjects in a low pressure chamber, both after  $\text{NH}_4\text{Cl}$ -administration the day before, and without it, the  $\text{O}_2$ -pressures of 149, 87 and 69 mm existing in the inspired air, corresponding to the heights of 0, 4,000 and 5,500 metres.

(3) The following results were obtained:

(a) HCl-secretion in 69 mm  $\text{O}_2$ -pressure was greatly reduced;  
(b) HCl-secretion was affected in 87 mm  $\text{O}_2$ -pressure too, but in a less degree and somewhat irregularly;

(c) HCl-secretion in all the  $\text{O}_2$ -pressures was independent of the preceding  $\text{NH}_4\text{Cl}$ -administration.

(4) The answer to our question seems to be — basing on the results — that the decrease of the HCl-secretion during severe  $\text{O}_2$ -deficiency is a consequence of anoxia, and happens independent of alkalosis produced by hyperventilation.

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## Microdetermination of pH in Saliva.

By

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The method here to be described is designed for determinations on freshly secreted saliva from the parotid and mandibular glands respectively. The separation is carried out as described below.

Saliva contains free carbon dioxide. When saliva is exposed to the air CO<sub>2</sub> is quickly lost, and the pH increases. In order to prevent the loss some investigators (*c. g.* BEOKS, 1924, WHITE and BUNTING, 1936, TENENBAUM and KARSHAN, 1937) have used paraffin oil as a protecting seal during the collection of saliva. LINDESTRÖM-LANG and HOLTER (1942) have shown, however, that the rate of CO<sub>2</sub> diffusion through paraffin oil is only four times slower than in water, and consequently the oil seal is not very effective as a protection against escape of CO<sub>2</sub>. Other investigators have made little or no effort to prevent the CO<sub>2</sub> loss. Most of the data in the literature for the pH of saliva are therefore, probably, too high.

Instead of paraffin oil the author used mercury as protecting seal. If saliva is sucked directly from the mouth into a syringe pipette, and the rest of the space of the syringe filled up with mercury, no CO<sub>2</sub> can escape.

Another source of error in determinations of the pH of the normal resting saliva is caused by variations in the composition of the secreted saliva due to the manipulations in sampling. If the collection of the saliva sample causes any stimulation of the glands, the pH of saliva increases markedly (as shown in the following paper). It is therefore important to choose a method

that requires as little saliva for the determination as possible, without reduction in the accuracy.

The colorimetric methods for pH determination are not very accurate especially not for microdeterminations. In applying electrometric methods the following electrodes are available: 1. the hydrogen electrode, 2. the quinhydrone electrode, 3. the glass electrode and 4. the antimon electrode. It is obvious that the hydrogen electrode with bubbling hydrogen cannot be used, because the bubbling through the saliva will wash out the CO<sub>2</sub>. Other forms of hydrogen electrodes could perhaps be used, but the use of hydrogen is always inconvenient. The glass electrode requires too much saliva. The ordinary forms require about 5 ml fluid, and it will be difficult to prepare an electrode that will require less than about 0.5 ml. The antimon electrode was used by STEPHAN (1940) for determinations of pH on the surface of the teeth with very satisfactory results. It seems that this electrode, in connection with a calomel bridge direct to the mouth, is very well suited for pH determinations inside the mouth. However the antimon electrode is not very accurate, so that when the determination can be carried out outside the mouth the quinhydrone electrode will be more favourable.

BÜLLMANN's capillary quinhydrone electrode is in many respects well suited for this special purpose. a) The volume of saliva necessary for a single determination is only 0.08 to 0.02 ml. b) The method is accurate when the pH to be determined does not exceed 8. The pH of saliva never exceeds this value. c) The presence of proteins does not produce any measurable error when the protein content of the sample is below 1—2 per cent. In saliva the protein content is about 0.5 per cent. d) When the concentration of salts is below 0.2—0.5 n, the salt error is negligible. The concentration of salts in saliva is about 0.02—0.04 n.

### Method.

#### Apparatus.

1. *Two rubber cups as used in Gore's saliva separator.* Fig. 1. When the rubber cups are applied to the mucous membrane surrounding the papillae of the parotid ducts the parotid saliva will drop out of the glass tubes like saliva from a *Pavlov* fistula in a dog.

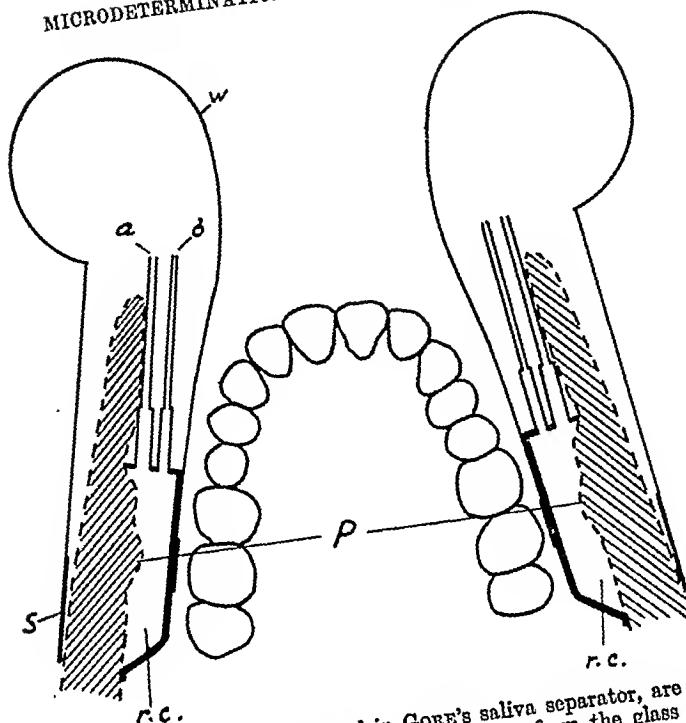


Fig. 1. Two rubber cups (r. c.), as used in GORE's saliva separator, are covering the papillae of the parotid ducts (P). The saliva drops from the glass tube (a). The glass tube (b) establishes connection with the atmosphere. A plate of stainless steel (s) is pressed by the elastic steel wire (w) against the outer side of the cheek just opposite the rubber cup, to keep it in position.

In order to transfer the saliva direct from the glass tubes to the syringe, the ends of the glass tubes as well as the tip of the syringe are ground down to be plane. When they are pressed together the saliva can be sucked directly into the syringe.

2. A syringe pipette (KROGH, 1935). The pipette is adjusted to contain about 0.15 ml. In order to reduce the dead space to about 0.02 ml the length of the tip is only about 3 mm.

3. A Büllmann capillary electrode. Fig. 2. The upper end of the glass tube (a) is ground down to be plane.

4. A calomel electrode.

5. A potentiometer.

#### Procedure.

The two electrodes are placed in a holder, the tip of both of them dipping into a vessel filled with a saturated solution of potassium chloride and connected with the potentiometer.

The syringe is washed with distilled water and the dead space filled with a suspension of quinhydrone in a little water. About 0.05 ml of a standard buffer solution (pH 6.00 or 6.50) is sucked into the syringe and the rest of the space filled up by mercury. The mercury fills out the dead space and a drop of mercury lies

in the lumen of the syringe. When the syringe is shaken thoroughly the mercury drop will serve for a complete stirring of the content of the syringe. When the stirring is finished, the syringe is held in a vertical position and the mercury is driven out, and now the mixture of buffer solution and quinhydrone shall be transferred to the electrode. The plane end of the small glass tube from the electrode (a. fig. 2) is pressed against the tip of the syringe and the mixture is driven out into the tube. The electrode is put together, placed in the holder and the adjusting of the potentiometer takes place.

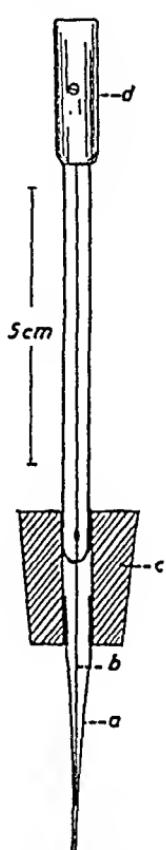
When the potentiometer is adjusted to the standard solution the determination on saliva can be carried out. The syringe is washed and the dead space filled with the suspension of quinhydrone. The saliva samples are taken in the following ways.

*The parotid saliva.* The person is sitting with the rubber cups (mentioned above) covering the papillae of the parotid ducts. The parotid saliva is dropping out of the glass tubes. The saliva nearest the outlet of the tube is sucked away with a piece of filter paper, the tip of the syringe is then pressed against the tube and about 0.05 ml saliva sucked into the syringe.

*The mandibular saliva* is sucked directly into the syringe from the floor of the mouth just under the tongue. In order to prevent loss of CO<sub>2</sub> the mouth must be kept shut until the moment when the sample is taken.

Fig. 2. A BILLMANN capillary electrode.  
a. Glass tube. b. Platinum wire.  
c. Rubber stopper. d. Contact for connection with potentiometer.

In a following paper the results of a series of determinations of the pH of normal resting saliva from the parotid and mandibular glands will be published. (All the measurements in this and the following paper are carried out at room temperature.)



## Experimental.

Some experiments to demonstrate the accuracy of the method are given here.

Table 1.

*Determinations on saliva samples with a quinhydrone electrode and a glass electrode.*

Sample no	Quinhydrone electrode	Glass electrode
1.....	7.00	7.01
2.....	7.07	7.07
3.....	7.17	7.15
4.....	7.22	7.22
5.....	7.28	7.29
6.....	7.42	7.48

In table 1 the results of pH-determination on different samples of saliva are given. The determinations are carried out both with a quinhydrone electrode and a glass electrode. The agreement between the results obtained with the two electrodes is seen to be satisfactory. (The results are higher than usual for saliva samples, but this is due to the fact that all the samples have been exposed to the air, and furthermore some of the samples have been collected during stimulation of the secretion.)

The effectiveness of a protecting paraffin oil seal has been tested in the following experiment: A saliva sample is divided into two equal parts each about 0.5 ml and placed in two small cylindrical vessels of 2.5 cm diameter. One of the samples is covered with a paraffin oil seal 5 mm thick. pH is determined in the two samples every ten minutes. From the results in fig. 3 it is seen that the oil seal affords a certain protection. During 50 min. pH only increases 0.18 pH units in the protected sample while pH in the other sample increases 0.32 pH units. But the fig. does not show the increase in pH during the collection of the saliva sample. The usual mode of proceeding

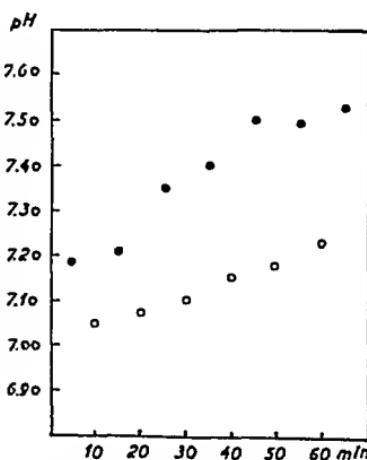


Fig. 3. pH variations of sample protected with paraffin-oil-(○) and unprotected sample (●).

for pH determinations of former investigators has been to let the saliva drop down from the mouth into a vessel containing a little paraffin oil. During this process some  $\text{CO}_2$  is lost. In one experiment a sample was collected in the manner described and pH determined. The value found was 7.01. A determination on the saliva taken by the help of a syringe directly from the mouth of the same person gave 6.72.

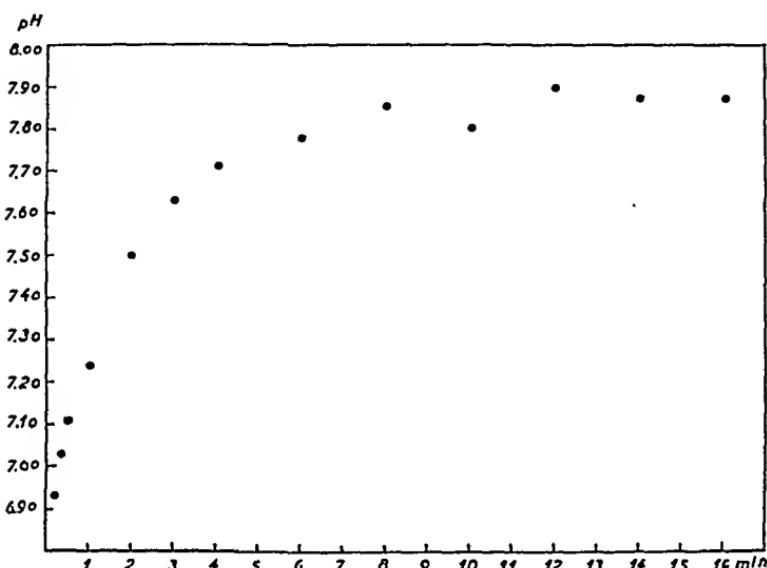


Fig. 4. pH variations of saliva sample stirred in an open vessel.

An experiment has been carried out in order to see how much the total loss of  $\text{CO}_2$  influences the pH of a saliva sample. A saliva sample is placed in a small open vessel and pH determined. The sample is stirred for 10 sec. and pH determined again. The procedure is repeated until constant values are obtained. The results are given in fig. 4. It is seen that after a total of 8 min. stirring pH reached a constant level. The corresponding increase in pH is one pH unit. But 50 % of the increase takes place in the first one and a half minute of stirring.

A series of consecutive determinations on two standard buffer solutions and a saliva sample have been made in order to test the accuracy of the method. (To avoid increase in the pH of the saliva sample during the determinations, the sample was stirred a little before the determinations and covered by paraffin oil.)

The results are given in table 2. It is seen that the accuracy is about 0.01 pH units.

Table 2.

*Consecutive determinations on two standard buffer solutions and one saliva sample.*

Standard buffer pH 6.50	Standard buffer pH 7.50	Saliva sample
6.50	7.49	7.08
6.50	7.49	7.08
6.50	7.48	7.09
6.50	7.49	7.08
6.50	7.48	7.09
6.50	7.48	7.10

### Summary.

A method is reported for determinations of pH in saliva from the parotid and mandibular glands. A micro quinhydrone electrode according to BIILMANN is used. A technique is described by which loss of CO<sub>2</sub> during both collecting and measuring of the sample is avoided.

Determinations are carried out to show the accuracy of this method in comparison with methods used by former investigators.

I wish to express my most hearty thanks to dr. HOLGER JÖRGENSEN, dr. H. USSING and dr. E. ASMUSSEN for valuable help and suggestions during the development of the method.

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## The pH in Parotid and Mandibular Saliva.

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In the preceding paper a micro method for determination of the pH in parotid and mandibular saliva was described (SCHMIDT-NIELSEN, 1945). A series of experiments have been carried out by this method. The purpose has been:

- 1) To find the conditions under which resting saliva can be obtained.
  - 2) To study the variations in pH of this saliva during a day and during a longer period.
  - 3) To compare the pH in resting saliva from a number of persons.
  - 4) To study the influence of stimulation on the pH of saliva.
- A large number of pH determinations in saliva are available in the literature, when examined they are found to be very heterogeneous. This is due to a number of factors as: inadequate methods for collection of saliva and determination of pH, CO<sub>2</sub> loss from the samples and stimulation of the secretion. Every single one of these factors is able to produce large variations in the results.

It is often difficult to judge the quality of the results, because the authors have given too scant information about the procedure and the experimental conditions.

In many cases the pH determinations were incidental to the main purposes of the investigation, and the authors have not considered their choice of method. The results have in a few cases been good, but in most of the cases they have been unsatisfactory.

Extensive lists of the older literature are given by BABKIN (1928) and ROSEMAN (1927 and 1932). A list of the newer investigations is given by BAŽANT and STUDNIČKA (1941).

### The Conditions under which Resting Saliva can be Obtained.

I have found that constant values for pH of saliva can be obtained only when the person studied is sitting completely quiet, every sort of talking, mastication, sneezing or coughing causes an increase in the pH of saliva. The increase depends on the intensity of the stimulus and is often remarkable. As an example can be mentioned that the pH in parotid saliva increased from 5.62 to 6.68 after a single short fit of coughing. In the beginning of an experiment the pH of saliva usually is somewhat increased (fig. 1). This is most probably due to the irritation caused by the rubber caps covering the outlet of the parotid ducts. The pH soon decreases to a constant level, and as shown in fig. 4 and 5

this level is very constant for the same person. When measuring the pH of resting saliva it is therefore necessary to repeat the determinations until a constant level is reached.

As every sort of stimulation of the saliva secretion causes an increase in the pH of saliva (shown in table I) it seems most probable that the lowest attainable value for the pH in saliva from a certain person is that nearest to the correct value for resting saliva for that person. Also the constancy of this lowest level indicates that the real value for resting saliva is attained.

Fig. 2 gives an example of how small the pH variations in parotid saliva can be in a period of 24 minutes, when the person is sitting very quiet. The pH is measured at intervals of 3 minutes. At the point marked by an arrow the person was told to do mastication movements, as seen in the fig. this causes a marked increase in pH.

As mentioned in the preceding paper sampling of the mandibular saliva involves suction by the syringe direct from the floor of the mouth. The touching of the mucosa, however, causes a

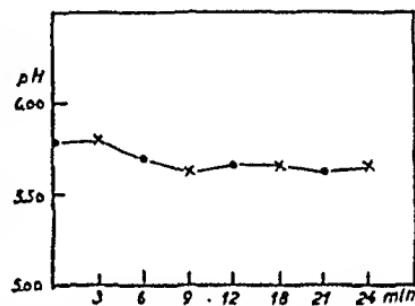


Fig. 1. An example of the increased pH in the beginning of an experiment.

- Saliva from the left parotid.
- × Saliva from the right parotid.

stimulation of the secretion with increase in the pH of the saliva. The increase has appeared not to last long. But if the samples are taken at short intervals (about 3 minutes) too high values will be obtained. Intervals of 10 minutes seem to be sufficient



Fig. 2. pH variations in saliva from one of the parotids during a period of rest. At the point marked by an arrow the person begins to do mastication movements.

to get satisfactory results. Fig. 3 gives an example of determinations in mandibular saliva. The first 7 samples were taken with intervals of 3 minutes, the later with intervals of 10 minutes. The results are definitely lower when the intervals of sampling are 10 minutes instead of 3 minutes. The average pH in the first samples is 6.84, in the last samples the average pH is 6.56. It is thus necessary, when pH in mandibular saliva is determined, to use intervals between samplings of at least 10 minutes.

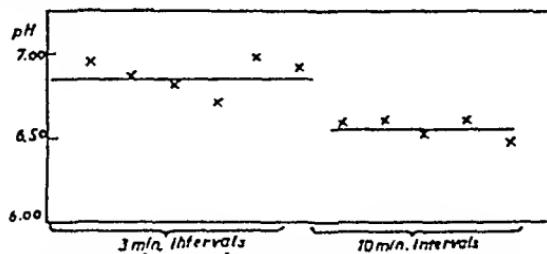


Fig. 3. pH in mandibular saliva determined with intervals of 3 minutes and 10 minutes.

### The Variations in pH in Parotid and Mandibular Saliva during a Day and during a Longer Period.

In fig. 4 the results of a series of determinations showing the variations during a day are recorded. The determinations were made on both mandibular and parotid saliva. Determinations were made on saliva from both parotids, but as the two parotids

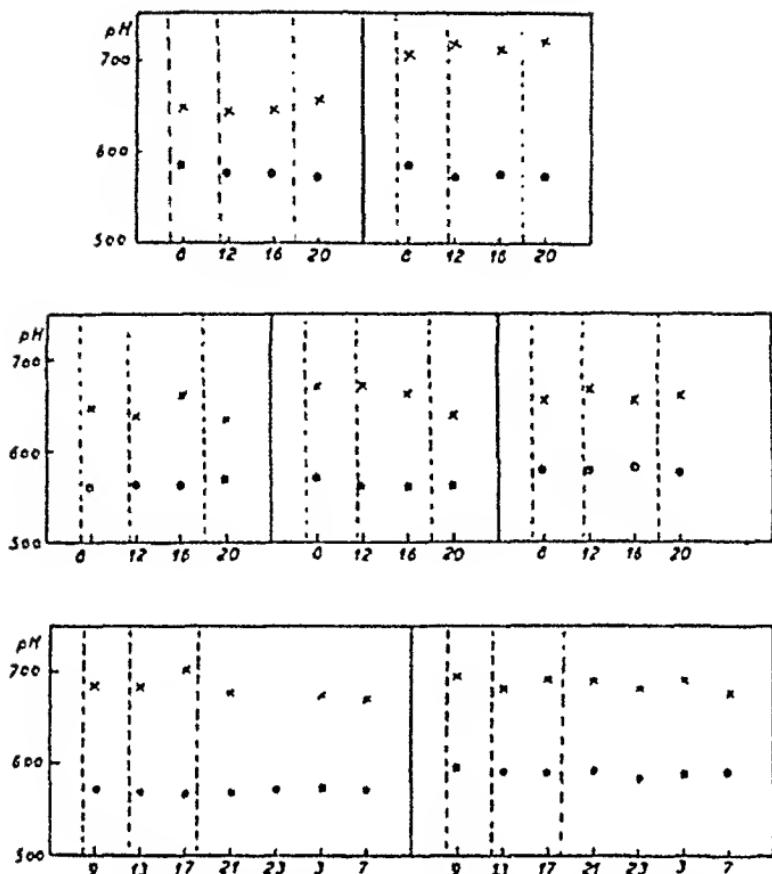


Fig. 4. The variations during a day in mandibular ( $\times$ ) and parotid ( $\odot$ ) saliva from 7 persons.

The vertical lines represent the hours of the meals.  
Abscissa, hours of the day.

always give nearly identical results, the values for pH in parotid saliva are only marked by one point in the figure. Five persons were investigated 4 times during a day, and two persons were investigated 7 times during 24 hours. The hours for the determinations and for the meals are marked in the figure. The two persons who were investigated in 24 hours went to bed after the sampling at 11 o'clock in the night. They rose for an hour for the sampling at 3 o'clock in the night, after that they slept again until just before the sampling at 7 o'clock. From the figure it is seen how remarkably constant the pH in the parotid saliva is for all the persons studied. The pH is independent of the meals and also of the time of day or night. The standard deviation for one person during a day is about 0.05 pH units.

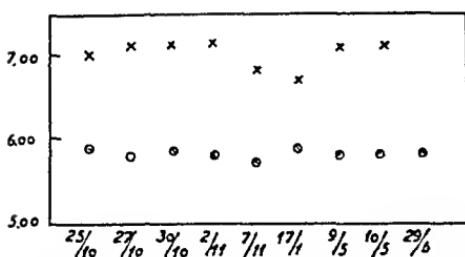


Fig. 5. The variations during nine month in pH of mandibular (X) and parotid (O) saliva from one person.

Abscissa, dates.

months are represented. Also in this case the pH of parotid saliva is remarkably constant, while the pH of mandibular saliva is more variable showing a standard deviation of 0.17 pH units.

### The pH in Resting Saliva from a Number of Persons.

The pH has been determined in resting saliva from 40 persons. The results are given in fig. 6. The 19 first persons are clinical assistants and students from the School of Dentistry, the remaining 21 persons are all pregnant women from the pregnancy consultation of the "Rigshospital".

It is seen that pH in the parotid saliva ranges from 5.45 to 6.06 (average 5.81). pH in the mandibular saliva ranges from 6.02 to 7.14 (average 6.39). There is, however, a systematic difference between the mandibular saliva from the pregnant women and the corresponding saliva from the other persons studied. The average for pH in mandibular saliva from the pregnant women is 6.22, while the corresponding average for the other per-

The pH in the mandibular saliva does not show quite the same constancy as the pH in parotid saliva. (This is probably due to the more difficult sampling where, as mentioned above, the sampling itself causes an increase in pH.)

In fig. 5 the results of determinations on saliva from one person during nine

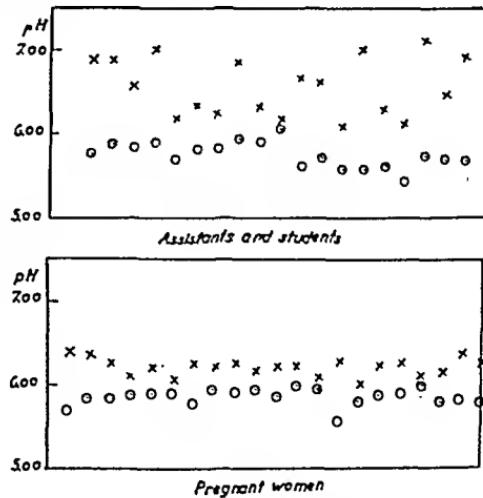


Fig. 6. The pH in mandibular (X) and parotid (O) saliva from 40 persons.  
Abscissa, the persons studied.

sons is 6.57. Furthermore the individual variations are definitely smaller for the pregnant women.

It is not possible to trace the cause of this difference from the material presented.

### The Influence of Stimulation upon the pH of Saliva.

The effect of three kinds of stimuli has been studied. These were: gustatory stimuli, olfactory stimuli and mechanical stimuli. Only the influence upon the parotid saliva has been studied, because the gustatory stimuli contaminate the mandibular saliva.

The procedure was as follows: First the pH in resting saliva was determined, then the stimulation was commenced and continued, coincident with determinations of pH, until a maximum in the increase of pH was reached. The gustatory stimuli: sugar, citric acid, salt and quinine were applied as crystals, which were placed on the tongue and distributed by small movements of the tongue.

In table I the results are given. It is seen that the gustatory stimuli have by far the strongest effect, but also the mechanical stimuli have marked effect while the olfactory stimuli have little or no effect.

Table I.

Gustatory stimuli	rest. sal.	stim. sal.	Olfactory stimuli	rest. sal.	stim. sal.	Mechanical stimuli	rest. sal.	stim. sal.
Sweet (sugar) ..	5.83	7.59	Ammonia damp. ....	5.81	5.88	Coughing ..	5.62	6.68
Acid(citric acid)	5.93	7.80	Formaldehyde damp.	5.81	6.07	Chewing ..	5.72	6.34
Salt (salt).....	5.83	7.52	Smell of chocolate.	5.70	5.80	Touching of the oral mucousa .	5.70	5.82
Bitter (quinine)	5.96	7.00						
Alkaline (so- dium bicar- bonate).....	5.96	7.50						

### Summary.

The author's micro method for the determination of pH in saliva (SCHMIDT-NIELSEN, 1945) is applied. It is found that:

1) Resting saliva can only be obtained when the person studied is sitting completely quiet. Even talking, mastication, coughing

or anything like that causes a marked increase in the pH of saliva.

2) The pH of resting parotid saliva is remarkably constant for the single person, not only during 24 hours, but also from day to day and from month to month. The pH of mandibular saliva does not show quite the same constancy, this is perhaps partly due to the technique employed.

3) The pH's of parotid saliva from 40 persons range from 5.45 to 6.06 (average 5.81). The pH's of mandibular saliva range from 6.02 to 7.14 (average 6.39).

4) Stimulation causes a considerable rise (up to two pH units) in the pH of the saliva. Gustatory, olfactory and mechanical stimuli were tried. Gustatory stimuli were found to have the strongest effect upon the pH.

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# Perception of Weight and the Phenomenal Regression to the "Real" Weight (Thing Constancy Phenomenon).

## Experiments on Arm-Amputated Subjects.

By

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In the visual field different constancy phenomena *e. g.* the constancy of visual size, shape, brightness and colour have been thoroughly investigated (for references consult *e. g.* BRUNSWIK (1934) or THOULESS (1931, 1932)). Similar constancy or invariance phenomena also exist in other sensory modalities, but in the field of weight perception the results of the investigations are, however, still somewhat unsatisfactory. This depends partly upon the fact that some results of the sensory muscular physiology have not been sufficiently taken into account, partly because the dual (tactile and proprioceptive) basis of weight perception has not been adequately considered. It seems thus necessary to discuss the peripheral physiology of the sensations of weight before dealing with the particular problem of the phenomenal regression to the "real"<sup>1</sup> weight.

The basis of weight perception varies both in similar experimental conditions between different subjects and in different conditions regarding the same person. As a general rule indications of the perception of weight are derived from skin receptors in all persons without exception when the lifted weight is small and

<sup>1</sup> For the meaning of "real" in this connection see a note by THOULESS (1931).



the skin would increase with the increase in the standard stimulus weight (according to WEBER's rule) it would, perhaps, at a certain weight level, be greater than the weight that would correspond to the muscular, proprioceptive difference threshold. In this case it seems quite natural that the receptive basis shifts from tactal to proprioceptive.

(The increase in the difference threshold would then stop until a further rise in the standard weight brings new muscles into action, thus tending again to raise the (proprioceptive) difference threshold (JALAVISTO (1935).)

Which are the phenomena of constancy or invariance in weight perception and how do they manifest themselves? Generally speaking the thing constancy can be defined in the following way: When phenomenal equality exists in spite of differences in stimulus conditions and this equality corresponds to a conceptual thing character e.g. the measured weight, there is conceptual invariance of the perception; in other words, the perception shows phenomenal regression to the "real" object. The main interest of this investigation is concentrated on the question of the existence of such a thing constancy phenomenon in the proprioceptive field, i.e. on the perceptions of weight based on impulses arising in the muscles and tendons. When *the perception of a weight sufficiently great to ensure a proprioceptive receptory basis of the sensation is not e.g. influenced by the arm weight there would be a constancy phenomenon of this kind.* It might be necessary to mention that if the weight to be lifted is small and the sensation of weight thus based on impulses arising in the skin and this perception remains uninfluenced by the arm weight, it certainly does not mean the existence of conceptual "thing" constancy. Earlier investigations dealing with the effect of arm-weight on the weight perception have not made this discrimination between tactal and proprioceptive cues in weight perception. KATZ (1920) e.g. stated, that a forearm amputated person who compared weights with his intact forearm and his amputated forearm (the moment of weight in relation to the elbow joint being the same) found that weights of 1,000 and 1,006 g were phenomenally of equal weight. Undoubtedly the comparison of weights is uninfluenced by the arm-weight in this subject, but the weight is not sufficiently great to ensure a proprioceptive nature of the sensations. FISCHEL (1926) again, when treating the same question, made his experimental subjects compare two weights, one of which was lifted with the arm in a water bath (the water

supporting the weight of the the arm), the other weight lifted by the other hand in an ordinary way in air. His experiments showed no influence of the arm-weight, but the same objection against his method as against that of KATZ is valid. The weight lifted in this case was also about 1,000 g and the perceptual basis of a weight of this magnitude is probably tactal as e.g. the investigations of WANGEL, ELMGREN, v. BAGH and RENQVIST seem to show. Other experiments by FISCHEL might, however, prove the existenee of a eentral invarianee phenomenon, but the perceptions are probably not proprioceptive. FISCHEL stated that a 1,000 g weight could be lifted in quite an arbitrary way: by teeth and arm, by one finger and four fingers, the arm loaded or without an extra load, and so on, but yet the weights were judged equal. FISCHEL explains the faet referring to the view put forward by MARTIN and MÜLLER, that the perception is based upon the impression of the absolute weight. The meaning of this is, however, quite identieial with the statement that the pereceptions show phenomenal regression to the "real" weight. PANZEL (1925) has made similar investigations, but his results were partly different. In his experiments the weight to be lifted was heavier, 2,000 g or more, and the muscular conditons, *muscular force* e.g. *influenced the sensation of weight*, but nevertheless the *arm-weight* had no effect. These experiments, made on two subjects, were arranged as follows: The weight was lifted alternatively with the arm flexed at the elbow joint, about 90 degrees, the weight of the arm acting against gravity, and alternatively with the arm supported in a horizontal plane, the arm-weight thus being compensated. In these experimental conditons it is, however, possible that the difference of the arm position in relation to the shoulder joint might have influeneed the result of the experiment. The length of the museles in these two positions cannot be considered equal, the biceps musele being shorter in an addueed position of the arm. Thus even if the arm-weight would tend to make the weight phenomenally heavier, the difference in musele length (that leads to an underestimation of the weight in the position whieh corresponds to the reduced muscular length) might have compensated and masked this effect so to say.

An other ease of phenomenal regression of the perception of weight towards a conceptual, thing eharaeter is suggested by KATZ and STEPHENSON (1937). When a weight is lifted by a string, the weight pereception corresponds to a great degree to the conception of elasticity. The sensation of weight is viz. influeneed in a greater

degree by the elastic properties of the string than by the muscle tension developed. In this case there need not be a question of a phenomenon of objectivation (phenomenal regression to a thing character), however, as it can be explained by the fact that the proprioceptive sensitivity to weight differences diminishes by shortening of the muscle. If the weight which is lifted without a string is lifted from the same end position that the arm reaches by stretching the string, no overestimation of the weight occurs. By this arrangement two weights lifted by and without a string are phenomenally equal (JALAVISTO, KALIN, PARVELA 1938).

Earlier investigations thus give no clearcut evidence of the existence of central constancy phenomena in the field of proprioceptive weight perception. It is true, that the well-known weight-size illusion can, according to THOULESS (1932) be treated in much the same way as e.g. visual constancy phenomena, but the receptive mechanism in the usual experimental arrangements is surely tactile. This phenomenon does thus not concern our problem. Especially the influence of arm weight on *proprioceptive* weight sensation requires further investigation. This paper deals with the special question whether it is possible to eliminate, so to say, the weight of the own arm, when the weight sensation is based upon the intensity of the muscle tension in the lifting muscles (perceptual indications being proprioceptive) and thus really perceive the "physical" weight. As the peripheral receptive mechanism shows great individual variations the experiments must be extended to a sufficiently great number of subjects. The investigation to be presented forms a part of an extensive study which concerns the psycho-physiology of the "phantom-illusion" of the amputated.

### Methods.

If an arm-amputated person is asked whether there is any phenomenal difference of weight between his amputated arm and the intact arm, the answer is sometimes that the amputated arm is lighter than the healthy arm. If the amputated arm is judged lighter, it seems evident that the weight of the intact arm influences the perception of the arm-weight. The perception of the weight of the own arm, in this case, certainly shows no "phenomenal regression to a thing character" or invariance: the sensation is consistent with the peripheral stimulus. This is, however, not the case when both the arms, amputated and intact arm, are judged equal. In this case it must be supposed, that the arm-weight is a part of the body image and as such a fixed conceptual

invariance and a difference or variation in the peripheral stimulus conditions (diminution of the arm-weight by the amputation) does not affect the weight sensation. As the indications for the perception of the own arm-weight cannot be but proprioceptive, this dual behavior of the amputated persons shows, that in some cases there is a central trans-formatory constancy, but in others the arm-weight sensation is dependent on differences in stimulus conditions. The result obtained in this simple way is, however, not quite reliable. The amputated arm is sometimes judged even heavier than the intact arm. This may be understood only by supposing, that a peripheral irritation in the arm stump influences the perception of weight quite as it molds the body image so that the "phantom arm" sometimes feels swollen, sometimes shortened or small, hot or chilly, and so on. As it is difficult to know in which case the peripheral irritation has influenced the perception of the weight of the own arm, one cannot rely solely on the phenomenology of the arm-weight of an amputated person, especially because the perception of weight of the own arm might possibly not have the same receptor basis as the perception of an external weight to be lifted. The question of conceptual constancy in weight perception must therefore be treated experimentally. The experiments are preferably to be made on amputated persons, so that the value of the beforementioned symptom: "lighter" — "of equal weight" may be settled at the same time. The result of such an investigation may be that always when an amputated arm is judged lighter, it may also be demonstrated that the weight of the subject's own arm influences the perception of an external weight and vice versa. In this case it is probable, that a peripheral irritation in the stump will not interfere with the sensation of weight of the own arm.

There are two ways, in principle, of eliminating the weight of the own arm.

1. The weight comparison is made between a weight lifted with the arm in a water bath, when the water supports the weight of the arm and a weight lifted in an ordinary way, the arm in air. In the latter case the weight must be judged heavier if there is no trans-formatory constancy and of equal weight if the weight sensation shows phenomenal regression to the "real" weight.
2. The weight comparison is made between a weight lifted in an arm position where the arm weight acts against gravity and a weight lifted by an arm hanging right down, the tissues passively supporting the arm.

The former experimental method is inconvenient, because, if the experiments are to be made with arm amputated subjects and the weight of the arm stump is to be eliminated, the water bath must reach over the shoulder of the subject. This is not possible with our laboratory conditions. The trouble with the latter method is, that to perform both lifting movements in exactly the same arm position which is necessary to avoid an influence of the muscle length on the sensation of weight, the subject must be tilted between lifting the two weights, the standard and the variable weight. This again is unsuitable, because

the up and down movement might have an irritating effect on the labyrinth. This method was nevertheless chosen, because it had some advantages compared with the former method and the irritation of the labyrinth seemed to be quite unimportant when the tilting was done slowly. Only 2 out of 54 persons became nauseous, the rest did not complain of any discomfort. On the other hand the underestimation of weight caused by an irritation of the labyrinth or tonic reflexes seems to be rather unimportant (ALLERS (1909), MANN (1912), HOLMES (1922), AIRILA and JALAVISTO (1939)).

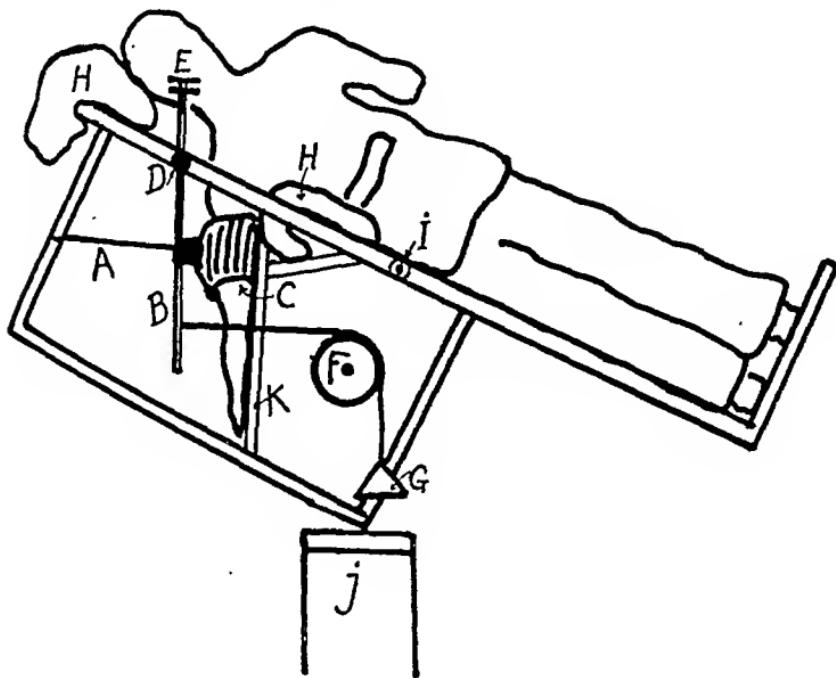


Fig. 1.

- A: Adjustable supporting rope.
- B: Lever with arm cuff.
- C: Pneumatic arm cuff.
- D: With ball bearings fixed axis of lever B.
- E: Counterbalance of the lever B.
- F: Pulley directing the action of weight G.
- G: 1,500 g (rotary moment at arm cuff 3,000 g).
- H: Soft cushions.
- I: Axis of the tilting mechanism.
- J: Stand to stop the tilting movement.
- K: Supporting board for the arm.

The supporting framework of the tilting mechanism is omitted.

The experimental subject was standing with his side against a tilting mechanism (Fig. 1) with the arm leaning against a support which formed an angle of 70 degrees to the vertical plane. The arm was flexed about 20 degrees in the elbow joint and abducted in the shoulder joint about 70 degrees. Above the shoulder there was in the ventrodorsal direction

a bar fixed with ball bearings and provided with a lever parallel to the arm. In the lever there was an adjustable arm cuff and a hook for the suspension of the weight. The suspending rope went over a wheel, so that the weight always pulled the lever in a right angle. In an opposite direction there was a supporting rope which prevented the weight from pressing the arm between the experiments, when the arm was held passive. The length of the rope could be adjusted so that immediately when the patient abduced his arm, the weight came to exert its whole weight on the arm. The same apparatus was suited for experiments both on right and left arm: the patient had only to turn 180 degrees round his vertical axis, and so the apparatus could be used for experiments with the other arm too. The inside of the arm cuff was bolstered up with a pneumatic rubber bag to ensure a proper fitting for every arm size. The soft pneumatic arm cuff is necessary also to avoid sensory impulses from the skin and deeper tissues to influence the weight sensation, thus favouring the proprioceptive basis of sensation.

The experiments were performed in the following way:

- a) *The main experiments:* The standard stimulus weight (3,000 g) lifted with the patient standing upright, the arm leaning towards a support, the arm 70 degrees abducted. When lifting the variable weight to be compared the subject lay in a tilted position of 70 degrees with his arm hanging right down. The subject has to judge whether the second weight (= standard weight + additional weight varied in steps of 100 g) lifted in tilted position is heavier, equal to or lighter than the standard weight lifted in upright position. An additional weight which in  $\frac{2}{3}$  of the cases gave the judgement "heavier" was considered as the difference threshold weight in these conditions. The experiments were performed both on the amputated and the intact arm.
- b) *As control experiments,* determinations of the difference threshold weight with the 3,000 g standard weight were made with the standard weight and the variable weight both lifted in the same erect posture of the subject, the arm abducted as in the main experiments. These control experiments too were performed with the intact as well as with the amputated arm. As a rule every additional weight (100, 200, 300 etc.) was tried ten times in the a) experiments, six times in the control difference threshold b) experiments.

The experiments were carried out on 27 arm and 27 forearm amputated war invalids<sup>1</sup> operated in the years 1941—42 and examined from, 1—12, most 8—9 months after the amputation. The number of the right- and left-hand amputations was in both groups nearly the same: among the arm amputated there were 14, among the forearm amputated 15 right-hand amputations. For technical reasons only persons with nearly the same and not too short a length of the arm stump could be selected for the experiments. Among the forearm amputated the differences in stump length were somewhat greater. The stump

<sup>1</sup> Patients at the Invalid Hospital of the Finnish Red Cross.

length was measured in all subjects, but the differences were not as great as to justify a grouping of the cases and separate treatment according to the stump length.

All the subjects were asked, in addition to the weight discrimination tests, whether the phantom-arm or the stump was phenomenally heavier, equal to or lighter than the intact arm.

## Results.

*Control Experiments. Weight discrimination in upright posture, the arm weight acting against gravity.* In the first place we had to test the difference threshold with a standard weight of 3,000 g in these experimental conditions. In some persons there was a so called negative time error, *i.e.* the same or even a lighter weight lifted after the standard weight was judged heavier. In these cases it happened occasionally, that the second variable weight had to be reduced to obtain perceptual equality. The difference threshold weight is then marked = 0 or negative. No difference between the stump or the intact arm concerning the magnitude of the time error could be observed. Fig. 2 shows the distribution of both the positive and negative threshold weights in different experimental groups. The ordinate represents the number of cases, the abscissa the difference threshold in grams. In the histogram the difference threshold weights in the experiments with the intact arm, the arm- and the forearm-stump are separately indicated. As can be seen the distribution of the difference threshold weight in all these three groups is quite similar. In every group the difference threshold weight is usually about + 100 g. This seems to indicate, that on the basis of difference threshold experiments the arm weight seems not to influence the weight perception. In Fig. 3 the *differences* between the threshold of the intact arm and the stump arm experiments are separately plotted. A negative value indicates, that a difference threshold weight obtained in stump arm experiments is greater than that obtained in the intact arm experiments on the same subject. If the weight of the arm were to influence the magnitude of the difference threshold, the histogram would be unsymmetrical, so that the positive values would be more numerous than the negative ones. The arm amputated cases would also tend more towards positivity than the forearm amputated cases. A tendency in this direction seems to exist between the arm and the forearm amputated cases, but the difference is too small to be of any statistical

significance. ( $\chi^2 = 2.7$ , when the degrees of freedom are 2.  $\chi^2$  is calculated from a table, in which the number of the negative,  $\pm 0$  and the positive difference threshold weights in the arm and forearm cases are indicated. The distribution does not differ significantly from a chance distribution, the probability for the distribution found being far above 5 per cent.)

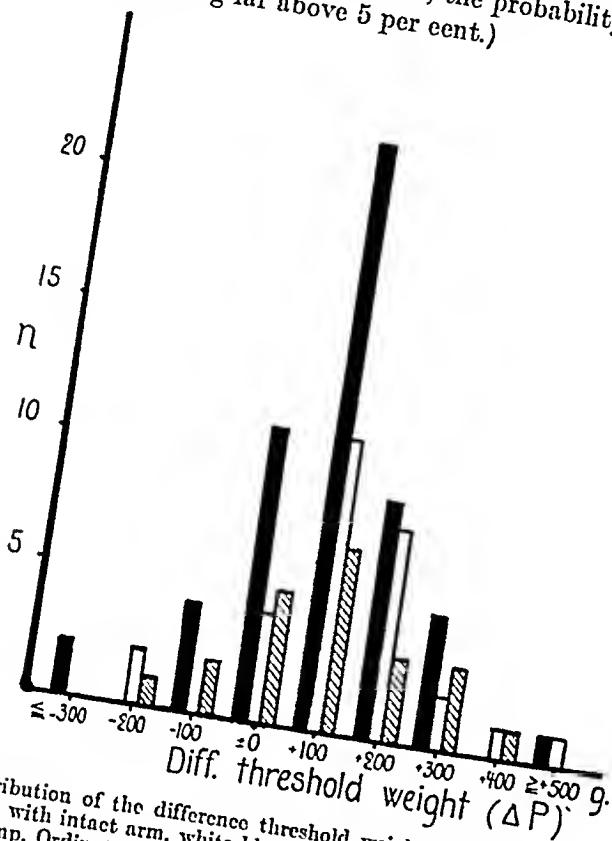


Fig. 2. Distribution of the difference threshold weights in control experiments (black blocks: with intact arm, white blocks: with forearm stump, striped blocks: with arm stump. Ordinate: number of cases, abscissa: difference threshold weight in grams).

As may be seen later on it would be incorrect to follow on the basis of these experiments, that the weight of the arm would not influence the perception of an external weight; it merely demonstrates, that the question cannot be settled in this way. This depends chiefly thereupon that in experiments on weight sensation WEBER's rule is not valid. (MERKEL (1889), JALAVISTO (1935), RENQVIST, v. BAGH and ELMGREN (1932) etc.). The fact that the difference threshold weight obtained in experiments on amputated

subjects is so often greater than in the intact arm experiments, shows, that there is no reason to believe that a possible influence could be masked by the inaccuracy of the threshold determination.

*Weight discrimination in upright and tilted posture, the arm weight alternatively compensated.* Before conclusions can be made from experiments in which the subject will repeatedly be tilted, the question must be settled to what degree and particularly in what direction the possible irritation of the labyrinth may influence

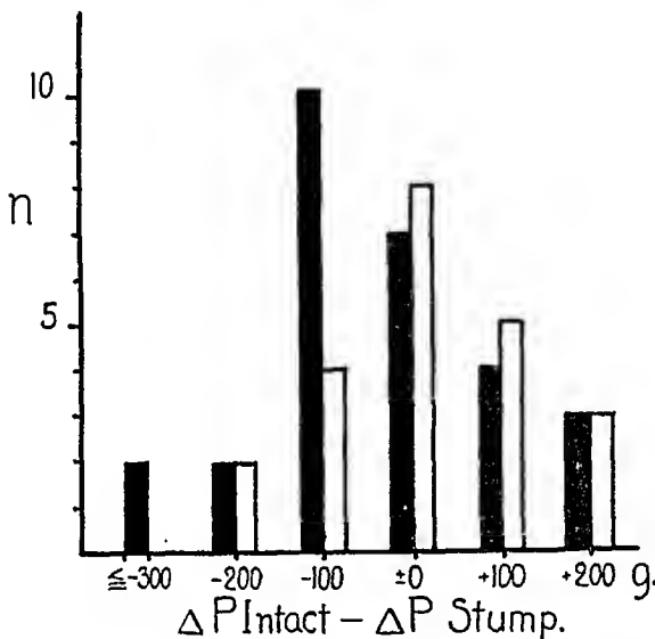


Fig. 3. Difference of the threshold weights in intact arm and stump arm experiments. Ordinate: number of cases, abscissa: difference threshold weight of the stump experiments subtracted from the intact arm difference threshold weight. (Black blocks: forearm stump, white blocks: arm stump experiments.)

the results. As the labyrinthine irritation in two persons was so strong that they became somewhat nauseous, it may be supposed, that the weight sensation in these subjects would be more altered than in those ones who did not complain of any discomfort. Both the nauseous subjects were overarm amputated cases. The results of the experiments performed on the arm stump are in these two cases opposites. In one of these subjects the weight to be lifted with the arm stump in the tilted position was lighter than the one lifted in the upright position. In the other, however, the weight lifted in the tilted position was phenomenally heavier, i.e. contrary to the expectation if the weight of the stump would affect the

weight sensation. The difference threshold was consequently negative and one of the biggest negative values ever encountered even in the stump experiments. The change in position and the possibly resulting labyrinthine excitation could consequently at most diminish the effect of the arm-weight, but certainly not enforce it. A similar negative difference threshold was, however, sometimes met with in control experiments in which there is no question of a labyrinthine effect, thus showing that the negative threshold in the nauseous subject may not have any significance.

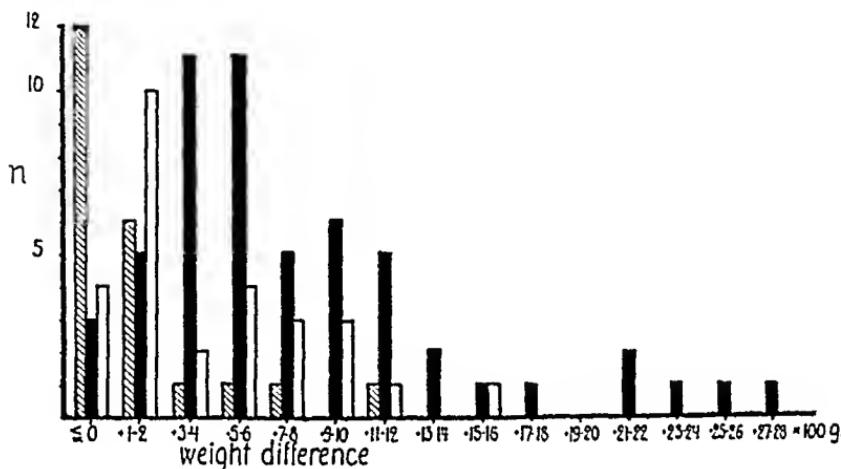


Fig. 4. Distribution of the "weight difference" in intact arm experiments (black blocks) in forearm stump experiments (white blocks) and arm stump experiments (striped blocks). Ordinate: number of cases, abscissa: "weight difference" in 100 g.

Generally in experiments with the intact arm hanging vertically, the elimination of the arm-weight causes an underestimation of the weight to be lifted, so that the weight difference corresponding to the difference threshold in the two unlike postures is rather big and positive. The additional weight of 2,900 g was the biggest one required to make the weight lifted in the tilting position just noticeably heavier than the 3,000 g standard weight lifted in the erect posture. Such a large difference occurred, however, rather seldom, amounting only in five cases to 2,200—2,900 g. (It may be noticed, that in all but one case the difference threshold weight determined in the erect posture (control experiments) was nevertheless of the usual 100—200 g magnitude.) Fig. 4 shows the distribution of the weight differences corresponding to the difference threshold. The weight difference shown in Fig. 4 (later on

simply called "weight difference") indicates *how much greater the additional weight must be in the tilting experiments than in the control experiments to make the variable weight phenomenally heavier than the standard weight.* (Thus:

$$\text{Diff. threshold} - \text{diff. threshold} = \text{"weight difference"}.$$

main exp.	control exp.
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The values thus show the effect of the tilting posture on the difference threshold independently from the possible difference in the magnitude of this threshold in the control experiments. As may be seen from Fig. 4 the difference in the experiments with the intact arm is usually 400—600 g (in 22 cases), seldom less (in 8 cases), but very often 700—2,800 g (in 24 cases). As the difference threshold weight is on an average  $79 \pm 97$  g, only differences greater than 300—400 g may be considered significant. According to the tilting experiments with the intact arm, the arm-weight seems thus to influence the weight perception in 35 cases out of 53 (one case is omitted owing to the abnormally high difference threshold weight of 900 g in control experiments). *In 19 cases no certain influence was observed.* If the results of these experiments are compared with similar ones performed with the stump arm, it can be seen, that the influence is real and not for example a labyrinthine effect. In case of experiments with a forearm stump the maximum of the "weight difference" has, as can be seen from Fig. 4, shifted only slightly to the left (smaller values), compared with the maximum of "weight difference" in the experiments with the intact arm. In the arm stump experiments, however, *the greatest number of cases show a difference of  $\leq 0$*  (these cases are summed up in one column), the maximum being definitely more to the left than in the other experimental groups.

Having established the fact, that the weight of the arm obviously influences the perception of weight in some cases, the question has to be settled, why this influence is lacking in as many cases as shown by the experiments. In the first hand it should be decided, whether there is a question of a central conceptual constancy phenomenon or whether the perceptual basis of the weight sensation in these persons, and regardless of the rather heavy standard weight, might be the intensity of skin pressure, in which case the arm-weight would not of course be expected to influence the perception of external weights. The question is not quite easy to settle, but the following discussion might give some indication of what sort of relation there is between the peripheral on central correlates

of the weight perception. If we examine the statements of the subjects concerning the phenomenal weight of the amputated arm compared with that of the intact arm (whether the amputated arm is heavier, equal to or lighter than the intact arm) these judgements should vary at random if the perceptual correlate is always peripheral and the receptory mechanism tactal. If, on the contrary, there is no difference in weight perception in the tilting and the erect posture, and this would depend on a central transformatory constancy, the distribution of the statements concerning the phenomenal weight would be quite definite: the statement "the stump arm lighter than the intact arm" should correlate with a lack of difference in weight sensation between tilting and erect posture and vice versa. A special consideration should be given such cases in which the phantom arm feels lighter, but there is no "weight difference". The least complicated explanation in this case would namely be, that the repressive basis is constantly peripheral, either proprioceptive, when the weight of the own arm is judged, or tactal, when the sensation of weight arises in connection with the lifting of an external weight resting on the skin. In this latter case, the muscle tension developed would of course in no way interfere with the sensation of weight. Fig. 5 shows the distribution of the statements "lighter" and "of equal weight" plotted against "weight difference" in experiments with the intact arm. The ordinate represents the number of cases, the abscissa the "weight difference". As may be seen, there is no quite firm relationship between the phenomenal weight of the amputated arm and the "weight difference" found in the tilting experiments. A clear tendency can, however, be observed that the statements "of equal weight" are more numerous in such cases in which the "weight difference" does not significantly differ from 0. When there is a weight difference, however small and on the limit of significance, *i.e.* 500—600 g, the number of statements "of equal weight" and "lighter" is the same. When on the other hand the "weight difference" is great, the amputated arm is more often lighter than the intact arm. If the cases with just significant "weight difference" are marked with 0, the cases without any "weight difference" with —1 and those in which the "weight difference" is obviously significant with +1, the correlation between the series can be calculated with the usual moment product method. The correlation coefficient thus obtained is  $0.42 \pm 0.15$  ( $= 2.8 \sigma$ ) and  $\chi^2 = 5.6$ . This means that the cases with no "weight

differencee" and those with a marked "weight difference", differ significantly according to the phenomenal weight of the amputated arm, because the probability that such a distribution might occur by chance is less than 2 per cent. In three cases only the weight sensation has a purely peripheral correlate as the judgement "lighter" corresponds in the main weight experiments to a lack of "weight difference" (black columns to the left from the striped line in Fig. 5). This correlate is tactful (skin pressure) in these experiments in spite of the rather heavy standard weight.

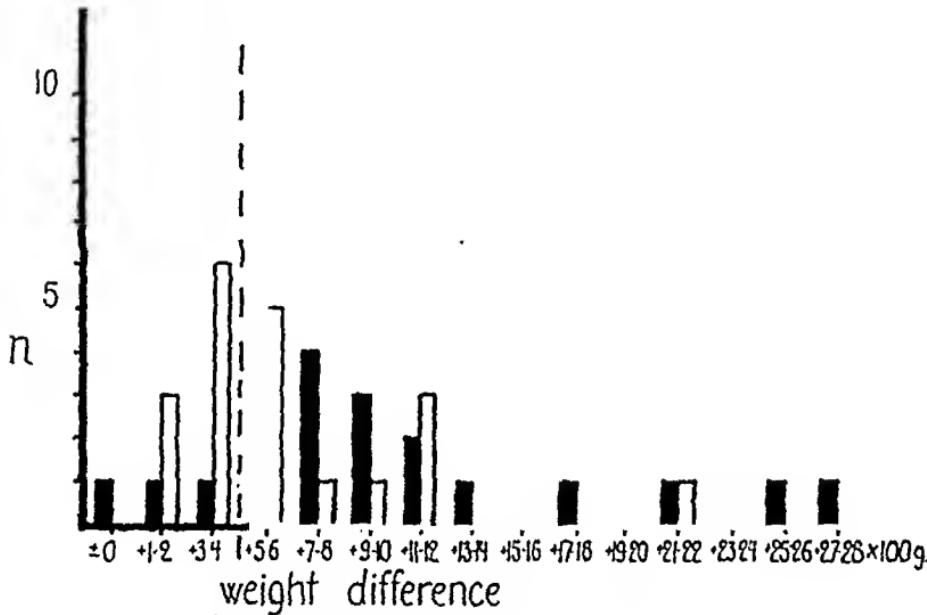


Fig. 5. Distribution of the "weight difference" in cases in which the amputated and the intact arm are judged of equal weight (white blocks) and in cases in which the stump is phenomenally lighter than the intact arm (black blocks). Ordinate: number of cases, abscissa: "weight difference" in 100 grams. To the left from the striped line the "weight difference" is not significant. (Striped line: border line of significance of the "weight difference".)

When trying to find out why the correlation between the judgements "lighter" and "of equal weight" and the "weight difference" is not more firm, the possibility must be kept in mind, that the state of irritation in the arm stump, which sometimes makes an amputated arm phenomenally heavier than the intact arm, may act in a way that the amputated arm which otherwise might be lighter becomes phenomenally of equal weight. It should also be noticed, that it is not always easy for the patient to judge the weight of his arms; sometimes the phantom arm is otherwise of equal weight, but if his attention is concentrated upon it and

especially if he swings his stump at the same time, the phantom arm begins to feel lighter than the intact arm. It may thus be possible, that the attitude of the different subjects when answering the question is not the same. On the other hand the real weight difference between the amputated arm and the healthy one is objectively smaller in the forearm amputated cases than in the arm amputated cases and it might be supposed that in the forearm amputated subjects this weight difference might be below the difference threshold of weight perception. This may be of no great significance, however, as in both groups of amputated, the cases, in which the weight of both arms is judged "equal" and the cases, in which the amputated arm is judged "lighter", are equally numerous.

### Discussion.

The results of my experiments differ partly from those of earlier investigations and partly they are consistent with them. Firstly, the arm weight seems very often to influence the weight sensation, when the weight to be lifted is great, contrary to what is suggested by KATZ (1920), in his experiments on amputated subjects and contrary to the results of FISCHEL (1926) and PANTZEL (1925) in their experiments on normal subjects. It is very probable, that this difference depends upon the fact, that in KATZ' and FISCHEL's experiments the indications for weight perception were impulses derived from the skin (in consequence of the small standard weight employed) and not from the muscles, as supposed by KATZ. It is true, that in some cases in experiments with amputated subjects that had Sauerbruch canalized muscles KATZ could show that the receptory basis really was proprioceptive, i.e. the weight corresponding to phenomenal equality was, when lifted with the canalized muscle, six times as heavy as a weight lifted with the intact arm. On this ground KATZ supposed that the perceptual basis would be the same even in different conditions and in different subjects. It is obvious, however, that the result cannot be generalized. KATZ' own experiments show, that in *other experimental conditions* and with *other subjects* the indications for weight perception cannot have been proprioceptive. Difference thresholds determined in experiments with the canalized muscle and those with the weight applicated on the forearm did e.g. not differ much from each other. The muscle tension corresponding to the dif-

ference threshold would, however, in the former case have been six times greater than the muscle tension corresponding to the difference threshold, when the weight was lifted with the healthy arm. The only explanation of this controversy might be that in experiments in which the phenomenal equality is determined (topological experiments by RENQVIST-REENPÄÄ (1936)), the perceptual cues might be the tension in the muscles, and in the experiments with determination of the difference threshold as cues might serve the impulses caused by the pressure exerted upon the skin. Considering the perceptual situation in both these cases this dual behaviour is quite comprehensible; the weight sensations may be qualitatively quite different owing to the altered sensibility of the canalization in experiments in which the weights to be compared are lifted alternatively by the intact arm and the canalized muscle. In this case it would be more adequate to rely upon the muscular tension, which most probably is a qualitatively uniform indication of weight, than upon the qualitatively differing cues of the tactile sphere.

In the ease of determination of the difference threshold, things are different: Both the standard stimulus weight and the variable weight are lifted in the same way, the sensations of weight being qualitatively similar in the two weight comparisons. Now there is no reason to abandon as indications of weight the tactile cues, which mostly are more accurate than the proprioceptive ones. The difference threshold weight consequently corresponds to the pressure exerted upon the skin and not to the tension developed in the muscles as in the ease of the topological experiments.

This shows how necessary it is to consider the receptive mechanism responsible for the weight sensation in every special experimental situation.

On the other hand my experiments gave evidence that in some subjects the weight perception based upon muscular impulses may be, so to say, controlled by a conceptual character which is demonstrated by the fact that the arm weight does not, in such cases, influence the perception of a lifted (external) weight. This behavior seems to be contrary to KATZ', FISCHEL'S and PANTZEL'S views, more uncommon than the weight sensation corresponding solely to the tension developed in the muscles.

The question arises: on what physiological process is the central invariance-phenomenon based? KATZ refers to the view suggested by G. E. MÜLLER (MÜLLER and SCHUMANN, 1889) that the weight

perception might depend on the motor impulse that effects the lifting of the weight. Thus a weight lifted with a greater impulse might be lifted with a higher speed and consequently judged lighter. According to KATZ the central nervous system might be so well adapted to the weight of the own arm, that the speed, with which the weight is lifted might, in all situations, be independent of the rotatory moment of the arm against gravity and the judgement of weight not invalidated by differences in it. Later on several investigators have shown that the judgement of weight is not influenced by the indirect evaluation of speed but is based on the direct perception of muscle tension or pressure on the skin (RENQVIST et al., for references see JALAVISTO 1935). Thus the adjustment of the motor impulses cannot explain the phenomenal regression phenomenon. As a matter of fact the question is difficult to solve experimentally as the main feature of the constancy phenomenon is precisely that, that a peripheral receptive correlate is lacking in a strict sense, and the only correlation to be found is the one between the weight sensation and a conceptual character of a "real" weight. In these experiments on lifted weights just as in other constancy (visual) phenomena (BRUNSVIK 1934, THOULESS 1931—32) the phenomenal character corresponds very often to an intermediate value between the stimulus and the conceptual character of the thing to be perceived.

### Summary.

1. The purpose of this paper was to investigate whether the weight of the subject's own arm influences the perception of an external weight to be lifted, when the receptive mechanism is proprioceptive (as is almost the case e.g. when lifting *heavy* weights).
2. The receptive mechanism of weight perception was briefly discussed.
3. The experiments were performed on 54 war invalids amputated on the upper limb. The experimental arrangement was as follows: The subject had to compare two weights, the one of which (a standard of 3,000 g) was lifted by the arm (the intact arm, or the stump) in an abducted position, the subject standing erect and the armweight acting against gravity, the other one (variable weight) lifted with the subject in a tilting posture, the arm hanging

vertically down and the tissues thus passively supporting the arm weight. The weight to be added to the variable weight in order to make the two weights phenomenally equal, showed great individual differences.

4. In experiments with the *intact arm*, in 19 cases, the additional weight was at the most 300—400 g greater than a weight that corresponds to the difference threshold determined the standard weight and the variable weight both lifted in the erect posture and the arm-weight acting against gravity. The difference is not statistically significant. In 11 cases it was 500—600 g greater, i. e. on the limit of significance, and finally in 24 cases 700—2,800 g greater, which difference is surely significant. In experiments with *forearm stumps* the corresponding figures were 16 : 4 : 8, and in experiments with *arm stumps* 19 : 1 : 2. It is obvious, that when experimenting with the whole arm, the *arm-weight influences the perception of an external weight in most subjects, in experiments on forearm stumps the influence is much less and in experiments on arm stumps hardly observable, quite in accordance with the fact, that the weight of the forearm stump is of course a little less, than that of the arm stump considerably less than the weight of the intact arm.*

5. Those cases in which (in experiments with the intact arm) the perception of a lifted weight was not affected by the arm weight (19 cases) proved, that the *proprioceptive weight perception shows phenomenal regression to a conceptual thing character analogous to the well-known constancy phenomena in the visual field*. In a few cases, however, there was reason to believe, that the receptive mechanism was tactile (pressure exerted upon the skin). In these cases the weight perception is of course independent of the arm weight.

Financial aid for this research has been granted by the KORDELIN foundation.

I hereby wish to express my gratitude to the Head of the Red Cross Invalid Hospital, Dr. REHNBERG for his permission to carry out the investigation on patients at the Hospital. It is a pleasure to record my indebtedness to the nurses and social case workers for their kind assistance in selecting and sending the patients over to the Physiology Institute. I am also grateful to Miss LIISA LAINE, for her valuable help and thoroughness in carrying out a part of the experiments.

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From Universitetets Biokemiske Institut, Copenhagen.

## On the Synthesis of Creatine in the Animal Body.

By

GUNNAR STEENSHOLT.

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In recent years the problems connected with the synthesis of creatine in the animal body have occupied the attention of a number of biochemists. As a result of the persistent efforts of these workers it can be said to-day, with a reasonably high degree of probability, that creatine is formed in the animal body by methylation of guanidino acetic acid, the methyl groups being furnished by suitable methyl donors among which in all probability methionine plays a very prominent part. The present writer recently had occasion to present a short review of these developments together with some new experiments which strongly corroborate the assumption just outlined of the mechanism underlying the biosynthesis of creatine.

It is a serious defect of much of the work done on creatine — and not only work connected with the biosynthesis of the substance — that the methods ordinarily employed for the quantitative determination of the compound are rather unspecific. This criticism also applies to the investigations of the present writer (STEEENSHOLT (1945)). It is, therefore, a matter of considerable interest to check previous work in this field by new and more specific methods whenever they become available.

Probably the most widely used method in studies on creatine is that of FOLIN (or one of its modifications), which is based on the so called reaction of JAFFE, *i. e.*, the fact that a red colour

develops when an alkaline solution of creatinine is treated with picric acid. This method was also used in the work of the present writer. Unfortunately, however, also other substances than creatinine, for instance glucose, react with picric acid, and this may seriously disturb quantitative determinations by this method. It is therefore of great interest that BENEDICT and BEHRE (1936) and LANGLEY and EVANS (1936) found in 3,5-dinitro-benzoic acid a reagent for creatinine with a fairly high degree of specificity. The workers mentioned found that the colour reaction of creatinine and 3,5-dinitro-benzoic acid is not disturbed by the presence of the following substances:

Glucose	Guanidine
Creatine	Methyl guanidine
Glycine	Dimethyl guanidine
Guanidine acetic acid	Fruuctose
Arginine	Cystine

Acetone and acetoacetic acid were found to interfere with the reaction. However, in work on muscles or muscle extracts these substances are hardly present in amounts sufficiently large to cause any difficulties.

As compared to the old method of FOLIN a method based on the new reaction would evidently be one of very considerable specificity. However, before a satisfactory procedure can be developed, two main obstacles have to be overcome, namely:

1) the violet-red colour which develops by the interaction between 3,5-dinitro-benzoic acid and creatinine in alkaline solution, is not very stable;

2) the 3,5-dinitro-benzoate solution has a rather strong colour of its own, very similar to that which develops in the presence of creatinine.

These difficulties, which necessitate special precautions, appear to have been successfully overcome by LEHNARTZ (1941) in a paper, in which he describes a method for the quantitative determination of creatine in muscle, based on the principles outlined above. The details of his procedure will be described below. Referring to the above remarks on the defects of the FOLIN method, it was considered advisable to check our previous results (STEENSHOLT (1945)) by means of the new method developed by LEHNARTZ. The present note is intended to give a report on the results of this work.

### Experimental Part.

*Biological material.* Our biological material consisted throughout of muscle and liver tissue from rats. The animals were usually from 4 to 8 months old, and were kept on a diet believed to be sufficient in all respects. They were killed by decapitation and the organs removed immediately after death. The tissue was placed on a watch glass and by means of a pair of bent scissors it was very finely divided into a homogeneous mass, which could be conveniently handled and weighed. In the experiments to be described below no difference was found between tissues from male or female rats.

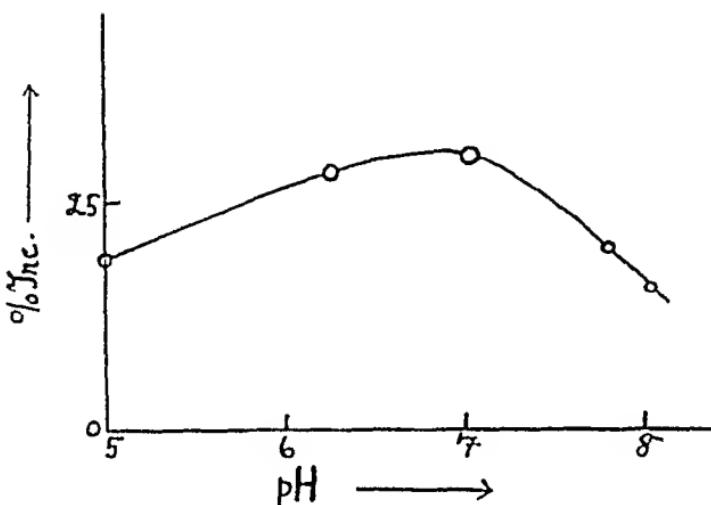


Fig. 1. pH dependence of the methylation of ethanolamine. Abscissa: pH; ordinate: relative increase (in per cent.) of choline in flask A.

*Experimental arrangement and method of analysis.* A typical experiment ran as follows: 0.3 g muscle tissue (from the hind legs of a rat) were suspended in 4 ml phosphate buffer (pH around 7.0), to which had been added 9 mg guanidine acetic acid and 50 mg methionine. This mixture was incubated at 37° C for 16 hours. At the end of this period 4 ml 20 per cent trichloroacetic acid were added for deproteinization, and in addition 1 ml 10 per cent hydrochloric acid. The mixture was centrifuged after 1 hour, and 5 ml of the supernatant liquid were autoelaved at 130° C for 30 minutes.

For the now following determination of creatinine the following reagents were used:

1. 6 per cent dinitrobenzoate solution, prepared in the following way: 30 g 3,5-dinitro-benzoic acid were suspended in 425 ml water under mechanical stirring. 75 ml 10 per cent  $\text{Na}_2\text{CO}_3$  were added and the stirring continued for 30 minutes until almost complete solution of the dinitro-benzoic acid. After filtration the solution was clear and of a faint yellow colour. The dinitro-benzoic acid was synthesised by the

writer by standard methods and purified by repeated recrystallisations from alcohol. A sample of commercial dinitro-benzoic acid was also available. After purification the two specimens behaved identically throughout the work.

2. 2.5 n NaOH.
3. 20 per cent sodium acetate solution.
4. Methyl red indicator.

After autoclaving the liquid was allowed to cool down. Some methyl red indicator was added, followed by sufficient sodium hydroxide to ensure exact neutralization. Water was then added to make the total volume of the liquid 11 ml. Then were added 10 ml dinitro-benzoate solution, 10 ml sodium acetate solution and finally 1 ml sodium hydroxide. After shaking the flasks were left standing for 5 minutes. At the end of this period their contents were brought quantitatively over into 50 ml flasks, which were subsequently filled up to the mark with water. The final colorimetric measurement was carried out in a Pulfrich photometer (using filter S 57). As comparison solution a solution was prepared in exactly the same way as above from dinitro-benzoate, sodium acetate and alkali.

A blank was carried out simultaneously on a reaction mixture consisting of 0.3 g muscle tissue and 9 mg guanidine acetic acid in 4 ml phosphate buffer. The working up of the mixture was, of course, exactly as described above.

On subjecting these two reaction mixtures to colorimetric measurement, we obtained in the first case the photometer reading 3.00 as the mean of five consecutive readings, and, similarly, in the second case the photometer reading 2.70. This indicates a very considerable increase in total creatinine in our reaction mixture as compared to the blank.

This procedure which was illustrated above by an example chosen at random from the laboratory journal, was systematically varied with respect to the relative amounts of tissue, guanidine acetic acid and methionine, and also with respect to the duration of the incubation, the amount of threecchloro-acetic acid and hydrochloric acid added, and the time of autoclaving. The results were qualitatively the same in all cases and further numerical details will therefore be left out.

By carrying out experiments such as the one just described in McIlvaine's phosphate = citrate buffer at varying hydrogen ion concentrations a pH-activity curve for the process was obtained. In the accompanying diagram we have plotted the relative increase in total creatinine expressed in per cent as function of pH. The main feature of the diagram is of course the fairly broad optimum somewhat above the neutral point. Several experiments of this type were carried out, but further numerical details are

left out, since it is felt that the diagram given is sufficiently illustrative of the general nature of the results.

Work on liver tissue gave similar result.

### Commentary.

On comparing the results presented above to those obtained by FOLIN's method and reported in a previous paper (STEENSHOLT (1945)) it is seen that both sets of measurements agree in a very satisfactory way. It may therefore be concluded that the results of the present paper strongly corroborate those previously given.

The writer is glad to express his best thanks to Professor EGE for his generous hospitality and support.

### Summary.

The paper describes the results obtained on applying the method of BENEDICT-BEHRE-LANGLEY-EVANS-LEHNARTZ for the quantitative determination of creatinine to the problem of the methylation of guanidine acetic acid in the animal body. It is found that muscle and liver tissue from the rat are capable of catalysing the methylation of guanidine acetic acid to creatine, the methyl groups being furnished by methionine. The results thus corroborate those of a previous paper.

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From Universitetets Biokemiske Institut, Copenhagen.

## On Methylation Processes in Etiolated Wheat Germs.

By

GUNNAR STEENSHOLT.

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For a number of years the methylation processes in living cells have aroused the keen interest of a number of biochemists. Probably the best known examples of processes of this kind are

- 1) the methylation of guanidine acetic acid to creatine; and
- 2) the methylation of ethanol amine to choline.

In both reactions methionine can function as methyl donator.

Most of the work in this field has been done on animal tissue (for a summary of much of the work see GUGGENHEIM (1940) and WERLE (1943); a very brief survey of recent contributions is contained in STEENSHOLT (1945)). Great interest was therefore aroused when BARRENSCHEEN and PANY (1942) reported some experiments which proved that etiolated wheat germs were able to transform guanidine acetic acid into creatine. The formation of creatine was found to be increased by a factor of 6 or even 8 on addition of methionine to the reaction mixtures. It was further found by BARRENSCHEEN and VALY (1943) that etiolated wheat germs transform glycine into betaine, methionine again acting as methyl donator.

These results have suggested to the present writer the desirability of investigating whether etiolated wheat germs are also able to methylate ethanol amine to choline in analogy with results recently obtained for animal tissue (STEEENSHOLT (1945)). The present note is a report on the results.

## Experimental Part.

*Biological material.* The wheat used was of three different kinds:

- 1) Jubilee wheat;
- 2) Svalöf Skandia wheat;
- 3) Record wheat.

The etiolation was carried out in the usual way by placing the germs on wet filter paper in the dark. The etiolated wheat germs were used after 6—7 days. They were first cut finely into pieces by means of a pair of scissors, and afterwards ground in a mortar. The resulting homogeneous mass could be conveniently handled and weighed.

*Determination of choline.* We decided to apply a colorimetric method, since this is probably the more convenient procedure for series determinations. For this purpose two methods are preferred today:

- 1) Determination as choline iodide (ROMAN (1930));
- 2) determination as choline reineckate (BEATTIE (1936)).

The method of precipitating choline as a reinecke salt is one of considerable specificity, and is probably the procedure most widely used to-day. The determination was usually carried out by measuring colorimetrically the reddish colour imparted to acetone by choline reineckate. However, only relatively concentrated solutions are sufficiently strongly coloured to yield accurate determinations. It may therefore be regarded as a considerable progress when ROSSI, MARENZI and LONO (1942) studied photometrically the method for determining the chromate ion ( $\text{CrO}_4^{2-}$ ) by a procedure based on the reaction of CAZENEUVE (1900), and found that it could be used for the determination of chromium in reineekates. On this basis MARENZI and CARDINI (1942) developed a new method for the determination of choline, which is claimed to be considerably more sensitive than those previously described in the literature. We therefore decided to apply the method of MARENZI and CARDINI in the present piece of investigation.

The technical details of the procedure are as follows:

### *Reagents.*

Saturated solution of ammonium reineckate in distilled water, prepared immediately before use. The concentration of the solution is approximately 4 per cent.

96 per cent alcohol.

100 per cent acetone.

60 per cent acetone.

10 per cent NaOH.

10 per cent (by volume) sulphuric acid.

0.2 per cent diphenyl carbazide in 96 per cent alcohol. This solution first acquires a faint rosy colour, which deepens after a few days. The solution can be used, nevertheless. The present writer worked with solutions which were never allowed to become more than 4 to 5 days old.

The amount of choline to be determined varies between 15  $\gamma$  and 100  $\gamma$ .

The volume of the sample to be analysed may range from 1 to 3 ml. The sample is placed in a centrifuge tube with slender end and an equal volume of a saturated aqueous solution of ammonium reineckate is added. The tubes are then cooled in ice water for at least 20 minutes. Longer cooling is, however, superfluous. The mixture is now centrifuged for 4 minutes (at 3,000 R.P.M.), and the supernatant liquid is afterwards removed as completely as possible without loss of precipitate by means of a fine tube provided with a suction bulb. The precipitate is washed with 0.5 ml ice cold alcohol two or three times. This operation must be carried out with care lest some of the precipitate be lost. The tubes are now again chilled for a few minutes in ice water and then centrifuged. The washings are repeated as described above. The supernatant liquid is now usually colourless, but sometimes a third washing has proved necessary.

The precipitate is now dissolved in about 1 ml of acetone and the solution is transferred to an ordinary test tube. The centrifuge tube is washed carefully 2 or 3 times with 1 ml 60 per cent acetone each time, and the collected washings are added to the solution in the test tube. We then add: 2 ml water, 0.2 ml sodium hydroxide and 0.1 ml perhydrol, for each 50 γ choline in the sample. Thus prepared the tube is placed in a boiling water bath. In the start the heating must be conducted carefully due to the rapid evaporation of acetone to begin with. After most of the acetone is evaporated the tubes are kept in the bath for 20 to 30 minutes, and finally they are heated over a naked flame for a few seconds in order to ensure complete elimination of the perhydrol.

During the heating the liquid acquires a yellow colour.

After the oxidation of the chromium has been completed the tubes are cooled and the contents diluted with 3 or 4 ml of water. 2 ml sulphuric acid are added together with sufficient diphenyl carbazide solution to give a final concentration of 8 per cent. The reaction mixture is finally diluted to an appropriate volume in a suitable measuring flask, in our work to 25 ml. The photometric measurements were carried out with the Pulfrich photometer, using filter S-53. The comparison tube contained a blank consisting of 2 ml sulphuric acid and 2 ml diphenyl carbazide solution made up to a final volume of 25 ml.

A calibration curve is conveniently used.

*Substrates.* The methionine was a Hoffman-la Roche product. The ethanol amine was synthesised by the writer according to KNORR (1897). The method of KNORR consists in leading a stream of ethylene oxide through a concentrated aqueous ammonia solution and subsequently fractionating the reaction mixture. This method was found to work very satisfactorily for the purpose of the present investigation.

A typical experiment was carried out as follows:

In a small flask A were placed

1 g plant tissue

0.200 ml ethanol amine

100 mg methionine

6 ml phosphate buffer (pH ~ 7.0).

A similar flask B contained exactly the same amounts of tissue and reagents but no methionine. Both flasks were incubated at 37° C for 12 hours. At the end of this period 4 ml 20 per cent triehloroacetic acid were added in order to remove proteins. After centrifugation 2 ml of the supernatant liquid were removed for choline analysis according to the method of MARENZI and CARDINI. Double analyses were always carried out. No difference in choline content between the two flasks could be found.

The relative amounts of tissue and reagents were systematically varied, as was also the time of incubation (up to 48 hours). Experiments were also carried out at 24°C. Qualitatively the results were the same in all cases; no difference in choline content between flask A and flask B could be found. The three types of wheat with which our work was carried out, behaved identically.

BARRENSCHEEN and PANY (1942) in their work on creatine synthesis in etiolated wheat germs found it necessary to oxygenate the reaction mixtures. It might therefore be supposed that oxygenation of the reaction mixture is required also for choline synthesis. Experiments to test this point were consequently carried out, but the results were identical to those just reported.

According to some previous experiments of the present writer, the methylation of ethanol amine to choline by animal tissue with methionine as methyl donor seemed to go somewhat better in McILVAINE's phosphate-citrate buffer than in other buffers. Accordingly this buffer was tried also for the purpose of the present work, but the results remained unchanged.

### Comments.

According to the experiments reported above etiolated wheat germs are unable to catalyse the methylation of ethanol amine to choline, with methionine as methyl donor. This is rather interesting in view of the results of BARRENSCHEEN and PANY (1942) already referred to, and shows that an enzyme or enzyme complex capable of catalysing the transfer of methyl groups from methionine to guanidine acetic acid is ineffective when ethanol amine is the acceptor. For animal tissue the corresponding specificity problem is still unsolved.

### Summary.

It is found that etiolated wheat germs are unable to catalyse the methylation of ethanol amine to choline, with methionine as methyl donator. The meaning of this is briefly discussed.

The writer is glad to express his best thanks to Prof. EGE for his generous support and hospitality.

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## Choline Esterases in some Marine Invertebrates.

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The presence of choline esterase (ChE) is necessary for any function of acetylcholine (ACh) and is, therefore, of great significance. ACh metabolism is, in itself, "intrinsically connected with the electrical changes during nerve activity, occurring everywhere of the neuronal surface" (BULLOCK and NACHMANSOHN 1942). From a comparative point of view, it is of interest to determine the ChE activity of different species of all animal groups; we want to know whether the ACh mechanism has been evolved parallel to the differentiated nervous systems.

This is the physiological point of the problem, which has already been investigated by several authors. A summary of these investigations is given below and in Table 1.

In the Protozoa neither ChE nor ACh are to be found. As a rule, Coelenterata do not show any ChE activity. In worms, considerable quantities of the ACh hydrolysing enzyme are present. The blood, however, seems to have no ChE activity. Among the Crustacea, particularly the lobster and the crayfish have been investigated, but other species have also been examined. The enzyme is to be found in muscle and nervous system, but is lacking in the blood. This is also the case with the spiders and the insects. Considerable amounts of ChE are found in the blood of the Mollusca. The activity is the lowest in the mussels (Lamellibranchiata), a little higher in the squids (Cephalopoda), highest in the snails (Gastropoda). High concentrations of ChE are found also in the muscles. ChE is lacking in the purple cyst of *Murex* (JULLIEN 1939; JULLIEN and BONNET 1941). It is present in the

Table 1.

Protozoa	MITROPOLITANSKAJA 1941; BULLOCK and NACHMANSOHN 1942
Cocleneterata	
Sponges	BACQ 1935, 1937 b; MITROPOLITANSKAJA 1941
Hydrozoa	BACQ and OURY 1937; MITROPOLITANSKAJA 1941; BULLOCK and NACHMANSOHN 1942
Scyphozoa	BACQ 1935; MITROPOLITANSKAJA 1941; BULLOCK and NACHMANSOHN 1942
Anthozoa	BACQ 1935, 1937 a; BACQ and NACHMANSOHN 1937; MITROPOLITANSKAJA 1941; BULLOCK and NACHMANSOHN 1942
Ctenophora	BACQ and OURY 1937; BULLOCK and NACHMANSOHN 1942
Worms	
Turbellaria	BACQ 1937 a; BULLOCK and NACHMANSOHN 1942
Trematoda	BACQ and OURY 1937
Cestoda	ARTENOW and LURJE 1941
Nematoda	BACQ and OURY 1937
Polychaeta	BACQ 1935, 1937 a; HALPERN and CORTEGGIANI 1935; BACQ and OURY 1937; BACQ and NACHMANSOHN 1937; RIECHERT and SCHNARRENBERGER 1942
Sipunculoidea	BACQ 1935 b, 1937 a
Crustacea	BACQ 1935, 1937 a; KOSCHTOJANTZ 1936; BACQ and NACHMANSOHN 1937; BACQ and OURY 1937; MARNAY and NACHMANSOHN 1937; JULLIEN and VINOENT 1938; ARTENOW and MITROPOLITANSKAJA 1938; NACHMANSOHN 1938, 1939; NACHMANSOHN and ROTHEBERG 1945
Spiders	BACQ and OURY 1937; CORTEOGIANI and SERFATY 1939
Insects	BACQ 1935; BACQ and OURY 1937; CORTEOGIANI and SERFATY 1939; TAHMISIAN 1941; MEANS 1942
Mollusca	
Gastropoda	BACQ 1935, 1937 a; GAUTRELET 1935; HALPERN and CORTEGGIANI 1935; AMMON 1935, 1943; KOSCHTOJANTZ 1936; BACQ and OURY 1937; VINCENT and JULLIEN 1938, 1939; JULLIEN, VINCENT, BOUCHET and VUILLET 1938; JULLIEN 1939, 1941; JULLIEN and BONNET 1941; REŽEK and HAAS 1942
Lamelli-branchiata	BACQ 1935; VINOENT and JULLIEN 1938, 1939; JULLIEN, VINCENT, BOUCHET and VUILLET 1938
Cephalopoda	BACQ 1935, 1937 a; BACQ and NACHMANSOHN 1937; JULLIEN, VINCENT, BOUCHET and VUILLET 1938
Echinoderma	BACQ 1935, 1937 a; BACQ and NACHMANSOHN 1937; BULLOCK and NACHMANSOHN 1942
Tunicata	BACQ 1935, 1937 b

blood, the muscles, and the nervous system of echinoderms. Tunicata have no ChE in the blood, but the muscles have an ACh hydrolysing activity.

The author of this paper has determined the ChE content of different invertebrates chiefly from a chemical standpoint.

The last four years of investigations of this enzyme have indicated that the ChE activity, in many cases, may not be attributed to only one enzyme; more types — 2 at least — are to be found.

In the case of these two ChE, we know with all probability that the enzyme in the erythrocytes is a specific choline esterase. On the other hand, there is no evidence in favour of a specific choline esterase in blood serum. This was first shown by VAHLQUIST (1935) in a cataphoretic investigation. Doubt that this "serum choline esterase" was identical with the acetylcholine hydrolysing enzyme in the erythrocytes was first expressed by ALLES and HAÜES (1940, 1941, 1944). This doubt was soon confirmed in different quarters. Thus RICHTER and CROFT (1942), MENDEL and RUDNEY (1943, 1944; see also MENDEL 1943; MENDEL, RUDNEY and STRELITZ 1944), ZELLER and BISSEGGER (1943), and NACHMANSOHN and ROTHEMBERG (1945) showed that ChE in blood serum and certain tissues was a non-specific enzyme. As regards the blood, definite proof has been established by this author (AUGUSTINSSON 1944, 1945) in a cataphoretic investigation. MENDEL and RUDNEY have named the ACh hydrolysing enzyme in serum "pseudo choline esterase", which has been described as an "unfortunate designation" (LAUBENFELS 1943).

All those papers have given rise to the assumption that we have to deal with two distinct choline esterases: one specific, and one unspecific ChE. The specific enzyme is found in erythrocytes (in some cases in the plasma; MENDEL, MUNDELL and RUDNEY 1943), in brain, and other nerve tissues, as well as in the electric organ of *Torpedo* (NACHMANSOHN and ROTHEMBERG 1945). LANGEMANN (1944) has, however, reported that the enzyme in the anterior pituitary lobe has not the same properties as that in the posterior lobe (and the erythrocytes). He also found the erythrocyte type in skeletal muscle (also NACHMANSOHN and ROTHEMBERG 1945) and, in certain cases, in the thymus. A mixture of the two types of enzymes is indeed present in most of the blood sera and many tissues, as well as in the superior cervical ganglion (MENDEL and RUDNEY 1943, 1944; MENDEL, MUNDELL and RUDNEY 1943). According to GLASSON (1944), a mixture is present in red corpuscles. The difference between the ChE in brain and serum has also been investigated by SCHÄR-WÜTRICH (1943).

Methods for estimations of the two types of ChE have been described by MENDELL, MUNDELL and RUDNEY (1943). These methods are based on the use of two different choline esters as substrates. Thus acetyl- $\beta$ -methylcholine is hydrolysed by the specific enzyme only, benzoylcholine by the unspecific (pseudo-ChE). Both types catalyse the hydrolysis of ACh. It is, however,

extremely doubtful whether such a test really is a demonstration of the whole point. That this doubt is justifiable, is obvious from the following investigations, described in this paper and others to follow. The results are obtained with different species of primitive animals.

### Methods.

Immediately after the animals were caught, they were frozen in at  $-20^{\circ}$  C. The frozen material was melted and minced. About 5 g. were taken for further grinding in a mortar with washed sand. The disintegrated tissue was then taken up with, in most cases, twice as much bicarbonate-Ringer solution ( $R_{30}$ ). The mixture was shaken for some minutes, centrifuged at a constant speed of 3,000 r. p. m., and the fluid decanted. Very different time intervals were needed to produce as clear a fluid as possible. The buffered suspensions were kept in the refrigerator, and the analyses were made, in most cases, the following day, in others, after two or three days.

The ChE activity was measured with the manometric method by WARBURG, in the same way as described in a previous paper (AUGUSTINSSON 1944). In the main compartment of the flask, 1.6 ml. of the solution of the substrate was placed; in the side bulb 0.4 ml. of the enzyme solution. For the dissolution of the substrates the same bicarbonate-Ringer solution ( $R_{30}$ ) was used, as in the preparation of the enzyme suspensions. The composition of  $R_{30}$  was: 100 ml. 0.9 % NaCl + 2 ml. 1.2 % KCl + 2 ml. 1.76 % CaCl<sub>2</sub> (cryst.) + 30 ml. 1.26 % NaHCO<sub>3</sub>. Fresh  $R_{30}$  was used for each experiment, since the solution deteriorates if kept.

The hydrolysis was carried out in a gas mixture of 95 % N<sub>2</sub> and 5 % CO<sub>2</sub>, and at  $37.5^{\circ}$  C. Readings were made continuously for 60 minutes for the period 5 to 65 minutes after addition of the enzyme to the substrate solution. The thermobarometer was filled with 2.0 ml. of the substrate solution with the same concentration as that in the reaction solutions.

Table 2.

Substrate	M	$R_{30}$ solution	ml. solution	Total conc. $v_F = 2.00$		a-b mean value
				%	molarity	
ACh .....	181.7	0.25 %	1.6	0.20	0.011	14
MeCh .....	195.7	"	"	"	0.010	3
BzCh .....	243.6	"	"	"	0.008	2

ACh = acetylcholine chloride (HOFFMAN-LA ROCHE); MeCh = acetyl- $\beta$ -methyl-choline chloride (MERCK); BzCh = benzoylcholine chloride (HOFFMANN-LA ROCHE).<sup>1</sup>

<sup>1</sup> The author wishes to acknowledge with thanks the gift of benzoylcholine chloride by Messrs. HOFFMANN-LA ROCHE.

The total volume of  $\text{CO}_2$  evolved during 60 minutes (enzymic + spontaneous hydrolysis) is expressed as  $a \mu\text{l}.$ , the enzymic hydrolysis as  $b \mu\text{l}$ . (total — spontaneous).

Table 2 shows the amounts and concentrations used of the substrates. The concentrations of the substrates were constant in all experiments and were all well above the optimum substrate concentrations in each case. The amounts of the enzyme preparations used are found in Table 3.

## Results.

In most cases no attempt was made to dissect the animals to find active tissues. 20 species of marine animals, belonging to 12 different groups of invertebrates, were examined.

The activity is expressed as  $b/100 \text{ mg}$  (Q in Table 3), that is  $\mu\text{l. CO}_2$  evolved in 60 minutes by 100 mg. tissues.

As regards the hydrolysing effect on ACh, these results confirm, on the whole, the observations made earlier. Strangely enough, the two worms did not show any ChE activity. With the Crustacea no unitary result was obtained. *Pandalus* and especially the muscles of *Carcinus* presented high activity; the intestines and the muscles of *Eupagurus*, on the other hand, were inactive (cf. BACQ 1935 a, 1937 a). A Placophora as well as two Gastropoda gave significant positive values. Large concentrations were found in *Dentalium*. The Lamellibranchiata seem to be less ChE active than the Gastropoda. Positive values were also obtained with a Brachiopod. Considerable amounts of ChE were shown in the whole animals of *Antedon petasus*. The other echinoderms were clearly ChE active. The quotients were very low for *Amphiura*, and among the Holothurioidea *Cucumaria* was about 5 times more active than *Mesothuria*.

In most cases, ACh was split at a higher rate than any other of the two substrates. This result is in accordance with "the main feature of choline esterase" (NACHMANSONN and ROTHENBERG 1945). In one case ACh only was split (*Terebratulina*). Of the two other substrates, only MeCh was hydrolysed in most cases, for *Patella* and the ampules of the podia of *Asterias* this effect was even more pronounced than with ACh. The same amplitude was found in *Mya*, *Amphiura*, and Holothurioidea. BzCh, also, was hydrolysed in some cases. The activity was particularly strong for *Tonicella*, and the intestines of *Asterias*, even stronger than with MeCh. The muscles of *Carcinus*, also, split BzCh.

According to Mendel and collaborators, the hydrolysing effect

Table 3.

Species	Test susp.		mg. tiss. used	ACh		MeCh		BzCh	
	g. tiss.	ml. R <sub>23</sub>		b	Q	b	Q	b	Q
Polychaeta									
<i>Aphrodit aculeata</i> ....	1.4	5.6	100	0	0	0	0	0	0
<i>Nereis virens</i> .....	8.9	17.8	200	0	0	0	0	0	0
Crustacea									
<i>Balanus crenatus</i> ....	2.5	5.0	200	4	2	0	0	0	0
<i>Pandalus montagui</i> ...	10.0	20.0	200	71	35	24	12	0	0
<i>Carcinus maenas</i> :									
muscles .....	5.0	10.0	200	298	149	73	37	67	34
<i>Eupagurus bernhardus</i> :									
intestines .....	2.5	10.0	100	0	0	0	0	0	0
muscles .....	0.7	2.8	100	0	0	0	0	0	0
Plaeophora									
<i>Tonicella marmorata</i> ..	1.45	2.9	200	91	46	13	7	46	23
Gastropoda									
<i>Patella vulgaris</i> .....	4.95	10.0	200	96	48	121	61	0	0
<i>Purpura lapillus</i> .....	5.0	10.0	200	171	86	25	13	8	4
Solenoconchae									
<i>Dentalium entalis</i> ....	0.6	2.4	100	134	134	14	14	45	45
Lamellibranchiata									
<i>Mya arenaria</i> .....	5.0	10.0	200	54	27	41	21	15	8
<i>Astarte sulcata</i> .....	1.1	2.2	200	28	14	8	4	0	0
Brachiopoda									
<i>Terebratulina caput serpentis</i> .....	2.0	4.0	200	84	42	4	2	0	0
Crinoidea									
<i>Antedon pectinatus</i> ....	5.0	10.0	200	451	226	139	70	0	0
Astroidea									
<i>Asterias rubens</i> :									
intestines, stomach.	2.9	5.8	200	113	57	46	23	62	31
ampulla, podia ....	4.05	8.1	200	48	24	59	30	0	0
Ophiuroidea									
<i>Amphiura chiajei</i> ....	3.9	7.8	200	18	9	18	9	0	0
Echinoidea									
<i>Psammechinus milarius</i> : intestines .....	1.45	2.9	200	144	72	81	41	0	0
<i>Echinus esculentus</i> : intestines .....	4.1	8.2	200	169	85	81	41	6	3
Holothurioidea									
<i>Mesothuria intestinalis</i>	9.6	19.2	200	75	38	64	32	0	0
<i>Cucumaria lactea</i> ....	2.5	5.0	200	349	175	299	150	0	0

in the material examined must, in most cases, be ascribed to the specific ChE. In some species, however, there should be a "pseudo ChE" too, characterized by its power of hydrolysing BzCh. It is, indeed, of little probability that we in these cases have to deal with only "two types", the properties of which are so different in different material (see below under the Discussion). The two types should separately be responsible for the hydrolysis of ACh. Thus we can expect to find about the same proportion between

the speed of the hydrolysis of ACh and the sum of speeds, obtained with the two other substrates. A comparison of this kind is found in Table 4.

Table 4.

Species	$Q_{ACh}/(Q_{MeCh} + Q_{BzCh})$
<i>Pandalus</i> .....	2.9
<i>Carcinus</i> .....	2.1
<i>Tonicella</i> .....	1.5
<i>Patella</i> .....	0.8
<i>Purpura</i> .....	5.1
<i>Dentalium</i> .....	2.3
<i>Mya</i> .....	0.9
<i>Astarte</i> .....	3.5
<i>Terebratulina</i> .....	21
<i>Antedon</i> .....	3.2
<i>Asterias</i> .....	1.1
" .....	0.8
<i>Amphiura</i> .....	1.0
<i>Psammechinus</i> ...	1.8
<i>Echinus</i> .....	1.9
<i>Mesothuria</i> .....	1.2
<i>Cucumaria</i> .....	1.2

This table shows that the ratio in question in some cases is very different in comparison to the others. Particular facts must be present. The results are hardly to be explained by the acceptance of only two choline ester hydrolysing enzymes.

In the following discussion I will try to present the facts which may be the foundation for further studies on this problem.

### Discussion.

ACh is an ester, the ester linkage of which in no way differs from a linkage of the same kind in other esters. Because of this, there is nothing remarkable in the fact that the enzyme or enzymes, hydrolysing ACh, also might split an ordinary ester, e. g. methyl butyrate. Thus a ChE need not necessarily be specific for choline esters. This does not mean, however, that serum ChE, for instance, may be identical with an ordinary esterase. There are many facts that argue against this, in the first place the inhibitory effect of various substances. We have, with all probability, an *unspecific* ChE in blood serum. Besides the ester linkage, ACh has a positive ammonium ion which, no doubt, is of great importance in forming the enzyme-substrate complex. It is to be assumed that it is at the linkage of this group to the enzyme that the specific ChE-inhibitors react.

A *specific* ChE is such an enzyme as hydrolyses ACh at a higher rate than any other esters, but it may fully split them. Thus the specificity for ChE is relative. The forming of an enzyme-substrate compound is controlled by the substituted ammonium ion. This positive group may be assumed to be attached to a negative group in the enzyme molecule (in the apo-enzyme component). The velocity of the hydrolysis is controlled by the reaction between the ester linkage of the substrate and the enzyme (the co-enzyme).

According to the nomenclature of BERGMAN (1942), ChE should be a heterospecific enzyme. With the presence of two active centra in the enzyme molecule, we may explain the fact that a substrate concentration above the optimum one has a depressing effect on the specific ChE. In this case both centra are attached to the same substrate molecule, low concentrations, however, to two molecules (cf. ZELLER and BISSEGGER 1943). This assumption is confirmed by the fact that strongly positive proteins (MENDEL and RUDNEY 1944) change the properties of the enzyme in such way that the characteristic inhibition by an excess of substrates disappears. This effect of certain proteins is most probably a neutralisation of the negative group in the enzyme, that group which combines with the positive N-group. This also explains the different properties of ChE in different tissues, and suggests the necessity of using pure enzyme preparations. Compare also the cataphoretic investigation by the author of this paper (1944, 1945).

The real chemical difference between the two types of ChE, the specific and the un-specific, is difficult to understand nowadays. We shall in all probability find this difference in the linkage of the enzyme to the positive N-group. And if we assume the enzymes to be conjugated of a co-enzyme and a apo-enzyme, the difference between the enzymes is the consequence of different apo-enzymes to which components the N-groups are linked. Probably, there are more than 2 different apo-enzymes. This is clear from the investigations published here and others being published in a subsequent paper.

### Summary.

1. A survey of the investigations, made earlier on the choline esterase content in invertebrates, is a preface to this paper.

2. The acetylcholine hydrolysing effect for different marine invertebrates has been investigated.

3. The acetylcholine hydrolysing effect is compared with the hydrolysis of two other choline esters, acetyl- $\beta$ -methylcholine and benzoylcholine, which other authors have suggested using in order to estimate the specificity of choline esterase. The results show that the method is insufficient. Thus some species hardly split either of the two substrates mentioned; this suggests the possibility of the occurrence of an enzyme which is still more specific as regards acetylcholine.

4. The properties of the choline esterases are discussed in the light of present knowledge.

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## Investigations of the Phosphatase Activity in Serum and Organs after Ligation of the Common Bile Duct in Dogs.

By

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It has long been known that obstructive jaundice both in man and, produced experimentally, in animals is accompanied by an increase in the serum phosphatase activity. In the course of time various hypotheses have been forwarded in order to explain this increase, but only three of them have gained common interest.

The first hypothesis was suggested by ROBERTS (1930, 1933), who presumed that the serum phosphatase activity is increased owing to the lacking secretion of phosphatase with the bile. This causes an accumulation in the blood. This hypothesis was forwarded on the basis of investigations on the phosphatase content of the bile, and it was supported, among others, by R. G. ANDERSON (1935), HERBERT (1935), FLOOD, GUTMAN and GUTMAN (1937) and GUTMAN, HOGG and OLSON (1940).

In the second place, there is THANNHAUSER's et al. (1938), assumption that the increase in serum phosphatase during jaundice is not a true increase in the amount of enzyme, but rather an activation of the phosphatase present, caused either by the formation of activating substances or by the lack of inhibitors. As the only support for this theory THANNHAUSER showed that icteric serum after mixture with normal serum causes an activation of the normal serum phosphatase, a result which CANTAROW (1940), was able to confirm, while other investigators failed to do so.

The third hypothesis was put forward by BODANSKY (1937) who stated that the increase in serum phosphatase activity as a consequence of biliary obstruction is due to an increased delivery of phosphatase from the liver tissue. BODANSKY set forth the view that variations in the serum phosphatase activity under pathological conditions must be ascribed to disturbances in the phosphatase-production or -secretion in the organ which is suffering in the respective case.

Among the hypotheses described above BODANSKY's explanation of the increase in serum phosphatase appears to be the most probable one. Since, however, the phosphatase content of the liver under normal conditions is not very high, a study of the phosphatase activity of the liver under icteric conditions will be necessary in order to decide whether this hypothesis can be regarded as well founded.

The following sections deal with the changes in the phosphatase content of the liver, of other organs, and the serum, which arise from a ligation of the common bile duct.

### Analytical Method.

The phosphatase determinations were performed according to a method developed partly on the basis of BODANSKY's (1933, 1937) procedure, partly after LUNDSTEN and VERMEHREN (1936).

0.1 ml serum or organ extract was applied; as substrate was used disodium- $\beta$ -glycerophosphate dissolved in an ammonia buffer, pH 9.95, containing Mg ions; 1 hour hydrolysis at 37°. The inorganic phosphate was determined colorimetrically in the Pulfrich photometer according to BRIGG's (1922) method.

### Reagents.

1. 0.9% sodium chloride.
2. Substrate: 1 gm disodium- $\beta$ -glycerophosphate, 7 ml n ammonium chloride, 13 ml n ammonia, 2 ml n magnesium chloride, filled up to 100 ml with distilled water.
3. 10% trichloroacetic acid.
4. An acid molybdenum reagent: 25 gm ammonium molybdate, dissolved in 300 ml dist. water + 75 ml conc. sulphuric acid, diluted to 200 ml with distilled water. Imperishable.
5. 20% sodium sulfite. Durable c. 1 month.
6. 0.5% hydrochinone, 2 drops of conc. sulphuric acid per 100 ml. Durable c. 14 days.
7. Standard phosphate solution: 0.4394 gm potassium phosphate (Sørensen) per 1,000 ml. Contains 0.1 mg P per ml.

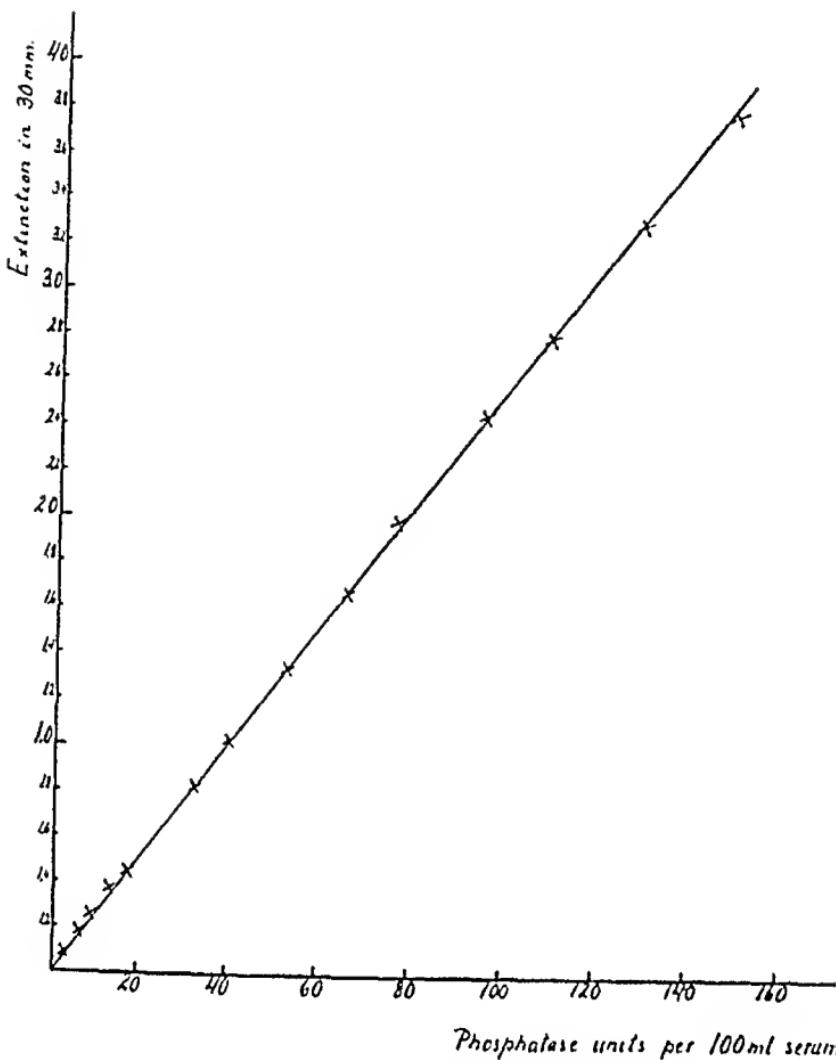


Fig. 1. Standardcurve for phosphatase determination.

1 ml serum (venous blood centrifuged for 20 min. at 3,000 rev.) was diluted to 100 ml with 0.9% NaCl. Into a 10 ml centrifuge tube 2 ml of the substrate (2) were pipetted; 1 ml of the serum dilution was added, the tube was closed with a stopper and was placed in the water thermostat at 37° for 1 hour; subsequently, the protein was precipitated by addition of 2 ml trichloroacetic acid (3). A control was made by adding immediately 2 ml trichloroacetic acid to serum dilution + substrate.

After  $\frac{1}{2}$  hour at room temperature, the precipitated protein was centrifuged off. From the supernatant 4 ml were pipetted off, 0.5 ml molybdenum reagent (4), 1 ml  $\text{Na}_2\text{SO}_3$  solution (5), 1 ml hydrochinone solution (6), and distilled water ad 10 ml were added. After 30 minutes, the colour intensity was measured in the Pulfrich photometer, filter S61, against a blank containing the same quantities of reagents as the analysis in 10 ml dist. water (Fig. 1).

An extinction curve was measured with known amounts of phosphate. No deviation from Lambert-Beer's law was found within the measuring range of the photometer.

The difference between the amount of phosphate in the analysis and in the control represents the amount of phosphate liberated by the phosphatase. The phosphatase unit is defined as the quantity of phosphatase which liberates 1 mg of phosphorus from the substrate in the course of 1 hour at 37°.

The determination of the phosphatase activity in organic extracts was performed on exactly the same lines as described for serum.

The extracts were prepared in the following way. 5 gm of fresh organ, if possible an average sample (freed from tendons, membranes, blood etc.), were ground with sand and extracted with 50 ml dist. water for c. 10 minutes and, subsequently, the extract was centrifuged. 1 ml of the extract was diluted to 10 ml with 0.9% NaCl. For the phosphatase determination 1 ml of this dilution was used in the same way as described for serum. Extracts which are especially rich in phosphatase, such as intestine- and kidney extract, were further diluted 10 times prior to the phosphatase determination, so that in no case more than 10% of the amount of glycercophosphate used was split, since the cleavage products formed during hydrolysis have some inhibiting effect if more than the mentioned 10 % of the total amount of the substrate are hydrolyzed (BODANSKY 1933, 1937).

This method for the production of organ extracts was completely satisfactory for the comparison of the phosphatase content in normal and in icteric organs.

The phosphatase activity was calculated from these comparisons for extracts which are 1% with regard to dry matter. The content of dry matter was determined on the first aqueous extracts before dilution with NaCl by drying to constant weight at 100°; the dry matter was always found to be between 1 and 2%.

The results of the phosphatase determinations obtained by this method can approximately be compared with those of BODANSKY (1933, 1937) since the definition of the phosphatase unit is the same. However, the present values appear to be somewhat higher, as I have used a more alkaline substrate and addition of Mg ions.

### Animal Material.

All the experiments were carried out on healthy and adult dogs.

For some time previous to and during the whole experimental period the animals were kept on a constant diet of black rye-bread and milk. No significant loss in weight could be observed in the course of the experiments.

### Experiments.

#### 1. Determination of Normal Values.

A series of determinations was performed of the phosphatase activity in serum and organs of normal dogs.

Table 1.

Serum	Liver	Pancreas	Kidney cortex	Spleen	Intestine (jejunum)
3.1	11.7				
1.9	3.9				
5.1	3.8				
3.9	4.2				
5.8	6.3	27.3	100		450
3.0	7.3	33.0	129		498
5.0	5.7	56.9	65		502
3.9	17.2	36.2	120		456
2.9	5.7	35.0	66		256
6.9	6.7	38.7	198		630
8.1	6.1	22.7	147	16.7	478
2.7	4.6	23.0	69	8.5	428
8.1	13.0	26.0	178	7.4	686
4.8	3.5	30.7	160	7.0	467
3.1	8.4	39.6	110		315
2.3	3.5	30.3	131	9.2	175

Phosphatase units per 100 ml serum and per 100 ml organ extract with 1% dry matter, pH 9.95, 1 hour hydrolysis at 37°.

The table reveals that the intestine contains by far the greatest amount of phosphatase; then follow the kidney cortex, pancreas, and finally liver and spleen, both showing rather low phosphatase contents. These results are in good agreement with those previously found (FOLLEY and KAY 1936).

The table shows, moreover, that the phosphatase activity in the same organs varies to some degree from one animal to another. However, the variation was not considered sufficiently large to prevent the application of this normal material being used as a basis for the comparison with the phosphatase values obtained after ligation of the bile duct.

## 2. Phosphatase Activity after Ligation of the Common Bile Duct.

Ligation of the common bile duct was carried out on 9 dogs. Before the operation, which was performed in morphine-ether narcosis, the serum phosphatase activity was checked for two days. In some cases, a piece of the liver was removed (an electrically heated thermo-cantery was used, no bleedings occurred) for a phosphatase determination simultaneously with the ligation. The icterus index was determined by means of MEULENGRACHT's method.

4 to 19 days after the operation the dogs were killed.

Table 2 shows the results of the determination of serum phosphatase activity and icterus index after ligation of the common bile duct.

Table 2.

*Serum phosphatase activity and icterus index after ligation of the common bile duct (dog).*

Dog No.	Before operation		2 days after operation		4 days after operation		7 days after operation	
	ict. ind.	phosph.	ict. ind.	phosph.	ict. ind.	phosph.	ict. ind.	phosph.
1. 9.7 kg		5.8		58.0		86.0		86.0
2. 27.0 "	2.5	5.4	10	35.5	33	109.0		
3. 23.5 "	3.5	3.4		48.0	48	77.5		
4. 19.0 "	3.0	1.9	25	22.0	35	78.0		
5. 30.0 "	2.0	3.9	48	12.6	58	54.0		52.0
6. 21.0 "	3.5	3.9	7.5	8.4	10	60.5	30	123.0
7. 10.0 "	3.0	3.5	32	44.0	30	106.0	32	141.0
8. 19.5 "	3.2	5.2	30	27.6	40	88.0	35	128.0
9. 21.5 "	2.5	2.9	30	67.0	42	119.0		

Phosphatase units per 100 ml serum. pH 9.95. 1 hour hydrolysis at 37°.

The table shows that, in the course of some days, the serum phosphatase activity increases simultaneously with formation of jaundice. The rate at which these changes occur is quite individual since, for example, 2 days after the operation the icterus index observed varies between 7.5 and 48, and the serum phosphatase activity ranges from 8.4 to 67 units. 4 days after the ligation the figures were found to be between 10 and 58 for the icterus index and between 54 and 119 units for the phosphatase activity, respectively. It is, furthermore, clear that the increase in the icterus index and in the serum phosphatase activity do not run parallel. The results are in good agreement with those obtained by other investigators.

With respect to dog No. 5 the experimental period was extended to 19 days. It was found that the phosphatase activity in serum reached a maximum of 116 units in the course of 10 days and then began to decrease again; after c. 3 weeks it had fallen to 46 units. Also these observations are in good agreement with earlier results.

Table 3 shows the results of phosphatase determinations on organ extracts after jaundice of various durations.

The table shows that bile obstruction has quite a different effect on the phosphatase activity in the organs. In the liver, for example, an increase to c. 10 times the normal value occurs (calculated on

Table 8.

*Phosphatase activity in serum and organs before and after ligation of the common bile duct (dog).*

Dog No.	Duration of icterus	Serum		Liver		Pancreas	Kidney cortex	Spleen	Intestine (jejunum)
		be- fore	after	be- fore	after				
4. 19 kg	4 days	1.9	78	3.9	68.5				
9. 21 "	4 "	2.5	119		48.0	33.5	224	19.4	1830
3. 23 "	5 "	3.4	77	9.7	60.2				
6. 21 "	7 "	3.9	123		102.5	51.4	200	8.5	650
7. 10 "	7 "	3.5	141		52.0	30.5	234	10.7	1350
8. 19 "	7 "	5.2	128		32.0		131	7.5	1465
5. 30 "	19 "	3.9	46	4.2	12.8	26.8	78		395
Normal values (Table 1)		1.9—8.1		3.5—17.2		22.7—56	65—198	7.1—17	175—686

Phosphatase units per 100 ml serum and per 100 ml organ extract with 1% dry matter. pH 9.95. 1 hour hydrolysis at 37°.

the basis of the average figures) during a short-lasting jaundice. In the intestine an increase of c. 300% was observed; measured in absolute figures this elevation is very remarkable.

In the case of pancreas and spleen no increase in the phosphatase activity content was found, while the phosphatase activity of the kidney may appear insignificantly increased.

The results obtained after long-lasting icterus (dog No. 5) indicate that not only the serum phosphatase as mentioned previously, but also the liver and intestinal phosphatase activities decreased after a maximum to almost normal values.

### Discussion.

It results from the experiments that the ligation of the common bile duct in dogs not only causes an increase in the serum phosphatase activity, a phenomenon which has been known earlier, but simultaneously produces an increase in the phosphatase content of liver- and intestinal tissues.

Some interdependence seems to exist between the phosphatase activity in serum and in these organs during obstructive jaundice.

The increased serum phosphatase may thus be ascribed to an overproduction of phosphatase in the liver, a hypothesis primarily assumed by BODANSKY 1937. It is, however, just as probable — or rather more probable — that the increased serum phosphatase during obstructive jaundice originates from the intestine which,

both normally and under icteric conditions, may give off its phosphatase to the liver, functioning as a kind of regulator for the phosphatase of the plasma.

More detailed investigations of the significance of both liver and intestine for the increase in the serum phosphatase activity after ligation of the common bile duct will be described in papers under preparation.

### Summary.

1. On the basis of BODANSKY's and LUNDSTEN and VERMEHREN's procedures an analytical method is described for the determination of the phosphatase activity in serum and organ extracts.

2. The phosphatase activity is determined in a series of organs of normal dogs. The intestine was found to have the highest phosphatase activity, then follow kidney cortex, pancreas and, finally, liver and spleen which both have rather low phosphatase contents.

3. The observations of other authors concerning the serum phosphatase activity after ligation of the common bile duct have been confirmed insofar as, a few days after ligation, a jaundice is observed which is accompanied by an increase in the serum phosphatase activity of up to c. 40 times the normal value. No parallelism is seen between the intensity of the jaundice and the serum phosphatase activity. Furthermore an increase in the phosphatase activity of the liver- and intestinal tissues is observed, while the stasis of the bile does scarcely affect the phosphatase contents of pancreas, spleen and kidney.

4. It is assumed that the increased serum phosphatase during obstructive jaundice may originate from the intestine.

I am indebted to cand. pharm. V. LARSEN for performing the operations of the dogs.

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## Studies on Serum Phosphatase Activity in Relation to Experimental Biliary Obstruction in Rabbits. II.

By

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In a previous publication (JALLING, LAURSEN and VOLQVARTZ, 1945) it was shown that the increase in the serum phosphatase activity in relation to experimental biliary obstruction may partly be explained by retention of phosphatase which is normally excreted with the bile, but besides a delivery of phosphatase from one or more organs may be of importance.

It is a natural thought, that the content of phosphatase is changed in the organ or the organs which deliver phosphatase to the blood. The experiments on rabbits which are presented in this paper show, that it is not possible to demonstrate any certain change in the phosphatase content of liver tissue or the intestinal mucosa at the moment when the increase in serum phosphatase after ligation of the common bile duct is known to be maximal. In the case of the renal cortex, on the other hand, it is possible to demonstrate a significant decrease in the phosphatase activity in connection with obstructive jaundice, as has been previously established by TAKATA (1932). This fall in the phosphatase activity of the renal cortex is without any causal relationship to the increase in serum phosphatase, the hyperphosphatasemia in animals with ligation of the bile duct and removal of both kidneys being accompanied by an increase in serum phosphatase of the same size as in animals where the kidneys had not been removed. Our determinations of the content of alkaline phosphatase in liver tissue

and intestinal mucosa have thus not brought us any nearer to the solution of the problem concerned with the serum phosphatase increase in connection with biliary obstruction in rabbits.

### Method for the Determination of Tissue Phosphatase.

KAY (1926) and MACFARLANE, PATTERSON and ROBISON (1934) have determined phosphatase in tissue using glycerocephosphatase as substrate. When their procedure is followed using disodium phenylphosphate as substrate no proportionality between amount of tissue used and phosphatase activity measured could be demonstrated. The following procedure was therefore worked out.

Immediately after the animal has been killed the organs in which the phosphatase content is to be determined are taken out. In case of the kidney the renal cortex is isolated. In case of the intestine 50 cm of this organ beginning 10 cm distally from pylorus are isolated and opened with a pair of scissors. The mucous membrane is rinsed with tap water and wiped and is then scraped off.

Portions of 0.25 to 0.5 g of the comminuted tissue are weighed out and ground with fine sand and a known amount of glycerol (87 per cent), which is added in small portions during grinding. This is continued until the mixture does not contain any visible tissue particles. The determination of phosphatase is carried out not later than two hours after the beginning of the grinding, and the glycerol extract is carefully stirred before adding samples of it to the buffer substrate solution. Phosphatase is estimated according to BUCH and BUCH's method (1939) for the determination of serum phosphatase, the Pulfrich photometer and a standard curve given by JALLING, LAURSEN and VOLQVARTZ (1945) being used. Buch and Buch's unit refers to 50 ml of serum, 0.25 ml of serum being used for the determination. *The phosphatase activity of tissue is calculated as phosphatase units per g of dry tissue dispersed in 50 ml of glycerol*, using 0.25 ml glycerol extract for the determination. The following example illustrates the procedure and the calculation of the phosphatase content in tissue.

*Rabbit No. 37.* Immediately after the death of the animal the liver is taken out. A part of the organ is comminuted. 8 samples of about 0.5 g are weighed out, and each sample is ground with sand and 20 g of glycerol. On each glycerol extract duplicate determinations are performed with 0.10, 0.15, 0.20 and 0.25 ml of extract. In order to get the same volume in each buffer-substrate-enzyme mixture 0.15, 0.10 and 0.05 ml of glycerol are added to the test tubes, to which are subsequently added 0.10, 0.15 and 0.20 ml of extract. In Table I (Columns 3 to 6) are given phosphatase units per 50 ml of diluted glycerol extracts, computed as the mean value of duplicate determinations. When the phosphatase activities found for 0.10, 0.15, 0.20 and 0.25 ml extract are plotted in relation to amount of extract used a straight line is obtained for each tissue sample; hence it is possible to compute the phosphatase activity

Table I.

*Determination of the Phosphatase Content in 8 Samples of the Same Liver Tissue.*

Sample No.	Liver tissue g	Phosphatase units per 50 ml of diluted glycerol extract				Ph. units per g dry tissue in 50 ml glycerol
		*0.10	*0.15	*0.20	*0.25	
1.....	0.4990	20.8	36.0	43.9	57.5	144.2
2.....	0.4997	21.0	37.5	45.3	59.0	149.4
3.....	0.4995	19.3	34.0	42.0	49.7	133.0
4.....	0.4995	21.0	33.3	39.9	54.4	137.8
5.....	0.4998	19.3	33.9	47.3	53.0	155.2
6.....	0.5003	22.0	33.4	43.2	53.5	141.2
7.....	0.4994	17.3	32.9	40.7	49.7	127.6
8.....	0.4993	22.8	36.1	40.7	55.3	143.4
Percentage of dry tissue:		26.20 %.				Average 141.48

\* ml of undiluted glycerol extract in 0.25 ml dilution.

of the tissue as the average of all determinations after having corrected for the dilution. The observed proportionality is also found for extracts of intestinal mucosa and kidney tissue when the following proportions are used: 0.5 g intestinal mucosa to 15 g of glycerol and 0.25 g kidney tissue to 30 g of glycerol. The phosphatase units per g of dry tissue dispersed in 50 ml glycerol = A is computed by insertion into the formula:  $A = 2 \times \frac{n \times (m + v)}{d \times v \times t}$ , where n = phosphatase activity per 0.25 ml

undiluted extract, a = amount of glycerol used in g, d = density of glycerol, v = weight of tissue in g, t = dry substance in percent, calculated by drying two samples of tissue to constant weight at 105° C.

It appears from Table I, that there is a fairly good agreement between the numbers of phosphatase units per g dry matter in 50 ml of glycerol calculated from the 8 samples of tissue.

In the experiments which are presented in what follows the phosphatase determinations are generally performed on 2 extracts from the same organ. On each glycerol extract duplicate determinations are performed on 3 different dilutions. The inaccuracy of the method has been determined on the basis of all the determinations which were carried out in duplicate or more. When the determinations for each organ were pooled the following coefficients of variability were found, liver tissue: V = 3.97 %, intestinal mucosa: V = 5.28 %, kidney tissue: V = 3.92 %.

### Experimental.

Our animal material consisted of normal rabbits and rabbits with ligation of the common bile duct. All the animals weighed between 2 and 3 kg. The controls had normal values of serum phosphatase, viz. 3 to 12 units, as was also the case with the operated

Table II.  
*Phosphatase Activity in Kidney Tissue.*

Normal rabbits				Rabbits with ligature of the common bile duct				
Rabbit No.	Kidney tissue			Rabbit No.	Serum ph. units	Kidney tissue		
	Dry matter %	* Ph. units	Average Ph. units			Dry matter %	* Ph. units	Average Ph. units
33	22.76	504.4 460.7	482.6	41	184.2	24.37	231.6 247.6	239.6
34	23.18	468.7 453.6	461.2	42	128.4	23.02	293.4 292.0	292.7
35	23.33	319.5 338.6	329.1	47	143.5	25.59	277.3 298.5	287.9
36	25.23	304.2	304.2				349.1	
37	25.38	310.4 314.0 364.2	312.2	62	135.6	22.05	334.4 320.9 320.4	334.8
60	22.80	359.0 365.4 467.8	362.9	63	132.0	21.82	335.5 329.8	328.6
61	23.46	502.3 495.9	488.7	65	99.5	24.53	155.9 151.4	153.7
64	23.07	447.2 452.2	449.7	68	142.5	23.40	338.9	327.6
66	25.80	418.2 399.5	408.9	70	115.5	23.66	291.5 272.7	282.1
67	24.35	466.4 443.3	454.9	72	116.3	21.24	258.1 266.0	262.1
69	21.51	311.9 307.9	309.9	74	93.0	24.18	327.1 328.2	327.7
71	23.65	387.1	387.1					

\* Ph = Phosphatase units per g of dry tissue dispersed in 50 ml glycerol

Normal rabbits: Mean value of ph.: 396.0;  $\sigma = 70.9$ .

Operated rabbits: Mean value of ph.: 283.7;  $\sigma = 55.7$ .

animals before the operation. The animals with ligature of the common bile duct were killed 17 to 24 hours after operation. As shown by JALLING, LAURSEN and VOLQVARTZ (1945) the increase in serum phosphatase after ligature of ductus choledochus is maximal 17 to 20 hours after the operation. Immediately before the death of the animal the serum phosphatase activity was determined, and the values found are given in Tables II—IV. In all the animals with ligature of the bile duct a marked jaundice was demonstrated. At section the gall bladder was found to be very distended. A few animals in which only moderate or no increase in serum phosphatase after the operation was found have been excluded from the material.

Table III.  
*Phosphatase Activity in Liver Tissue.*

Normal rabbits				Rabbits with ligature of the common bile duct				
Rabbit No.	Liver tissue			Rabbit No.	Serum ph. units	Liver tissue		
	Dry matter %	* Ph. units	Average Ph. units			Dry matter %	* Ph. units	Average Ph. units
37	26.20 (See Tabl.I)	141.5	34	163.5	27.70	211.8	211.8	
38	26.20	72.6	35	143.8	21.28	71.5	66.9	69.2
		72.3					78.3	
44	27.62	172.7	41	134.2	23.38	75.7	77.0	
		159.6				88.4		
		41.7	62	135.6	24.34	88.5	89.3	
60	25.28	40.2	40.7			91.1		
		40.2				103.1		
		53.8	63	132.0	24.27	108.4	105.2	
61	26.34	56.1	54.2			104.1		
		52.8				45.2		
64	28.69	68.3	67.2	65	99.5	47.5	46.4	
		66.0				140.9		
66	28.72	74.3	75.6	68	142.5	131.8	136.4	
		76.8				67.9		
67	28.53	66.2	67.8	70	115.5	67.4	67.7	
		69.4				68.0		
69	26.03	81.1	83.7	72	116.3	66.5	67.3	
		86.2				55.1		
71	28.34	82.6	83.9	74	93.0	59.9	57.5	
		85.1						

\* Ph. = Phosphatase units per g of dry tissue dispersed in 50 ml of glycerol.

Normal rabbits: Mean value of ph.: 85.3;  $\sigma = 38.8$ .

Operated rabbits: Mean value of ph.: 92.6;  $\sigma = 49.3$ .

In the animals determinations of the phosphatase content have been performed on kidney tissue, liver tissue and intestinal mucosa according to the method described above and the results are recorded in Tables II, III and IV.

It is remarkable that the phosphatase activities in the examined tissue from liver and intestinal mucosa vary extremely from one animal to the other. The standard deviation is somewhat smaller in the case of kidney tissue. At the time where the hyperphosphatasemia is maximal there is no doubt that the phosphatase activity in the renal cortex of the animals with obstructive jaundice is smaller than in the control material. Assuming the validity of the zero-hypothesis and calculating the common  $\sigma = 64.6$ , the value  $t = 4.03$  is obtained. From Fisher's Table of  $t$  it is seen that

SERUM PHOSPHATASE ACTIVITY.  
Table IV.  
Phosphatase Activity in Intestinal Mucosa.

Rabbit No.	Normal rabbits			Rabbits with ligation of the common bile duct			
	Mucosa			Rabbit No.	Serum ph. units	Mucosa	
	Dry matter %	* Ph. units	Average Ph. units			Dry matter %	* Ph. units
48	13.63	218.8 207.2 107.9	213.0	41	134.2	10.53	251.3 249.5
49	13.89	123.1 229.9	115.5	42	128.4	10.54	204.1 165.3
50	9.88	250.3 127.4	240.1	40	162.7	12.44	186.2 144.6
51	18.21	138.4 385.3	132.9	47	143.5	10.07	352.7 349.7
52	14.40	387.6 107.0	386.5	65	99.5	18.29	266.2 266.2
54	17.49	112.1 120.7	113.3	68	142.5	14.24	212.2 216.7
55	16.06	117.0 127.0	119.2	70	115.5	15.74	41.2 39.7
64	16.56	113.6 148.8	148.4	72	116.3	13.47	284.8 290.5
66	15.80	147.9 226.1	217.0	74	93.0	13.00	114.2 114.8
67	16.48	151.6 101.1	149.7				
69	15.46	94.7 146.4	97.7				
71	16.39	144.4	145.4				

\* Ph. = Phosphatase units per g of dry tissue dispersed in 50 ml of glycerol.  
 Normal rabbits: Mean value of ph.: 173.3;  $\sigma = 81.3$ .  
 Operated rabbits: Mean value of ph.: 208.9;  $\sigma = 97.0$ .

$P < 0.001$ . It is therefore justified to assume that the difference is significant.

It is natural to set the fall in the phosphatase content of the renal cortex in relation to the increase in serum phosphatase. Under pathological conditions a change in the permeability for phosphatase of the cells of the kidney might occur, and the fall in activity which is stated in the renal cortex may roughly correspond to the increase observed in serum. That phosphatase from the kidney is, however, of no importance to the increase in serum phosphatase after ligation of the common bile duct has been es-

tablished by the following experiments. On Rabbits 37 and 38 double-sided nephrectomy was carried out in connection with ligation of ductus choledochus. The values of serum phosphatase are to be found in Table V.

Table V.

*Serum Phosphatase in 2 Rabbits after Ligation  
of Ductus Choledochus and Doublesided  
Nephrectomy.*

Rabbit No. 37		Rabbit No. 38	
Time after operation hours	Serum phosphatase units	Time after operation hours	Serum phosphatase units
2 $\frac{1}{4}$	33.0	3 $\frac{1}{2}$	82.8
7 $\frac{1}{2}$	73.5	7 $\frac{1}{4}$	102.5
11 $\frac{1}{2}$	98.2	12 $\frac{1}{4}$	131.1
14 $\frac{1}{2}$	143.5		
18 $\frac{1}{2}$	159.0		
22 $\frac{1}{2}$	162.0		

It is seen that the rise which can be observed in the rabbits is of the same order of dimension as in rabbits in which the kidney has not been removed (cf. JALLING, LAURSEN and VOLQVARTZ, 1945).

TAKATA (1932) has stated that the  $\beta$ -glycerophosphatase content in kidney and liver from rabbits with experimental obstructive jaundice was lower than in normal rabbits. The animals were killed 48 hours after ligation of ductus choledochus (serum phosphatase determinations were not carried out). Whereas the difference between the phosphatase contents in kidneys from operated animals and controls is significant, TAKATA's material does not allow any conclusion with regard to the liver. TAKATA maintains that the lowered phosphatase contents in organs in connection with obstructive jaundice may be partly ascribed to the cholic acids which have entered the blood circulation, injections of sodium cholate subcutaneously being able to diminish the phosphatase content, which beforehand had been decreased by obstructive jaundice. In our opinion, however, the question concerned with the low phosphatase activity in the kidneys of rabbits with obstructive jaundice needs further examination, as we have no information about which kind of cholic acids are accumulated and their concentration, in the kidneys from operated animals.

The determinations of phosphatase in liver tissue and intestinal mucosa show that the values for these tissues from rabbits with obstructive jaundice lie within the range of normal values. There is no doubt that liver and intestinal mucosa, perhaps other organs too, contain so much phosphatase that they are able to deliver phosphatase to the blood, without any change being demonstrated in the organs by means of the experimental technique employed. The large spreading in the organ phosphatase content complicates the solution of the question further. It must at present be considered out of the question that a real increase in the phosphatase content of liver and intestinal mucosa should occur in immediate connection with ligation of the bile duct in rabbits.

### Summary.

The studies concerning the mechanism of the development of hyperphosphatasemia in rabbits with obstructive jaundice have been continued.

1. A method is described for the quantitative determination of phosphatase in tissues.

2. By means of this method determinations of phosphatase have been carried out on liver tissue, intestinal mucosa and renal cortical tissue from normal rabbits and rabbits on which ductus choledochus has been ligated. 17 to 20 hours after the operation, when the hyperphosphatasemia is maximal it is not possible to demonstrate any difference in the phosphatase contents of liver tissue and intestinal mucosa. In renal cortex a significant fall in phosphatase has been demonstrated.

3. The investigations do not give any explanation of the hyperphosphatasemia in rabbits in connection with obstructive jaundice.

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## The Presence of a Substance with Sympathin E Properties in Spleen Extracts.

By

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Since the discovery by LOEWI in 1921 of the liberation of an adrenaline-like substance on stimulation of the accelerator nerves of the heart evidence has accumulated to show that probably all adrenergic nerves owe their effect to some special substance produced or liberated at the endings of these nerves. As to the active principle liberated from the heart, or obtained in extracts thereof, LOEWI found that it conformed in its biological actions and chemical properties with adrenaline. On the other hand, the work of CANNON and his co-workers (1937) on the biological actions of "sympathin" from various sources appears to show quite conclusively that the mediator substance on several occasions is not adrenaline.

The problem merits special interest in view of the numerous reports dealing with the occurrence of blood pressure raising extracts of various organs, apart from the adrenals, the hypophysis and the kidney, where specific vaso-active substances are responsible for the actions.

The first observations seem to originate from OLIVER and SCHÄFER (1895) who found pressor actions in aqueous spleen extracts after a primary fall in blood pressure. Their observations were confirmed by BINGEL and STRAUSS in 1909. VINCENT and SHEEN (1903) have also observed pressor actions in organ extracts, and later such actions have been reported by ROGER (1922) and JAMES, LAUGHTON and MACALLUM (1926) in liver extracts. In an extensive study COLLIP (1928) was able to show that extracts

## SYMPATHIN E PROPERTIES IN SPLEEN EXTRACTS.

from a number of organs contained pressor activity in small quantities. The active substance was soluble in a mixture of acetone and ether, comparatively stable to acids and alkali and antagonized by cocaine. In extracts of the prostate gland a pressor substance has been demonstrated which agreed well with adrenaline (COLLIP, 1929, EULER, 1934).

HARTWICH and HESSEL (1932) found pressor activity in spleen and kidney autolysates which is probably due to tyramine (GRABE, KRAYER and SEELKOPF, 1934, EULER and SjÖSTRAND, 1943).

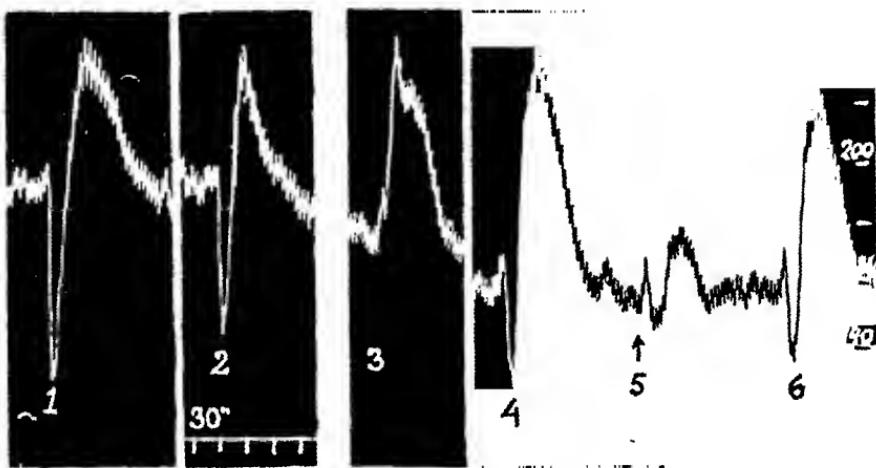
WILLIAMS and GROSSMAN (1938) found in perfusates of kidney an adrenaline-like pressor substance which they call perfusin, and ENGER (1942) has described the occurrence of a pressor substance, called nephrin, different from renin or hypertensin (angiotonin) in kidney extracts and in blood from certain types of hypertension. It has in certain respects sympathomimetic properties but differs obviously from adrenaline. It is considered to be specific for the kidney. Using colorimetric methods RAAB (1943) has found large amounts of adrenaline-like material in spleen extracts.

Of substances related to stimulation of sympathetic nerves LOEWI found (1936) in extracts of frog's heart an effect closely resembling that of adrenaline as to its action on the frog's heart and giving fluorescence in strong alkali like this substance (GADDUM and SCHILD 1934). The amount corresponded to 1—2 µg adrenaline per g of frog's heart. Stimulation of the sympathetic nerves did not increase the yield. In similarly conducted experiments on mammalian heart the effect corresponded to 0.1—0.2 µg adrenaline per g of fresh tissue.

GADDUM and KHAYYAL (see GADDUM, 1936) have observed that stimulation of isolated nerves containing adrenergic fibres liberated a substance which stimulated the frog's heart and in this respect was adrenaline-like. In extracts of adrenergic fibres or organs containing such fibres CANNON and LISSÁK (1939) and LISSÁK (1939) found a substance which was assumed to be identical with adrenaline.

Any substance having the properties of sympathin E does not seem to have been detected previously, however.

In continuation of the work of this laboratory on vaso-active substances in body organs and fluids with special reference to their behaviour in hypertension, it seemed of importance to investigate whether sympathomimetic pressor substances could be prepared from fresh organs. In a preliminary note (EULER, 1945)



*Fig. 1.* Blood pressure, cat, chloralose.

1. Extract 0.125 g cattle spleen.
2. 0.25 g of same, treated with N NaOH at 100° C for 10 minutes.
3. (After 10 mg/kg cocaine hydrochloride i. m.) 0.5 µg adrenaline.
4. 0.06 g of extract 1.
5. Same, treated as in 2.
6. 0.2 g of same.

it was announced that extracts from a variety of organs — except placenta — contain unexpectedly high amounts of pressor activity of a kind similar to that of adrenaline. The present paper is concerned with some experiments made in greater detail with extracts from spleen which was specially rich in the pressor substance.

### Experimental.

#### 1. Preparation and testing of extracts.

Extracts were made from fresh spleen of cattle. To the ground tissue 2 volumes of ethanol and 2 ml 10 N H<sub>2</sub>SO<sub>4</sub> per kg tissue was added and the mixture left for 1—2 hours in room temperature under occasional stirring. After filtering and evaporation of the filtrate to a small volume fatty material was removed with ether. When tested on the cat's blood pressure under chloralose the aqueous extracts caused a fall in blood pressure followed by a more or less conspicuous rise (Fig. 1). Pre-treatment of the animal with ergotamine tartrate (Gynergen)<sup>1</sup> in a dose of 0.1 mg per kg generally made the pressor response appear much more distinct. This effect is due to the exclusion of the mechanical presso-regulation mechanism (EULER and SCHMITTERLÖW, 1945). Sometimes vagotomy increased the pressor responses. Rabbits were unsuitable

<sup>1</sup> Kindly put at my disposal by Messrs Sandoz A.-G., Basel.

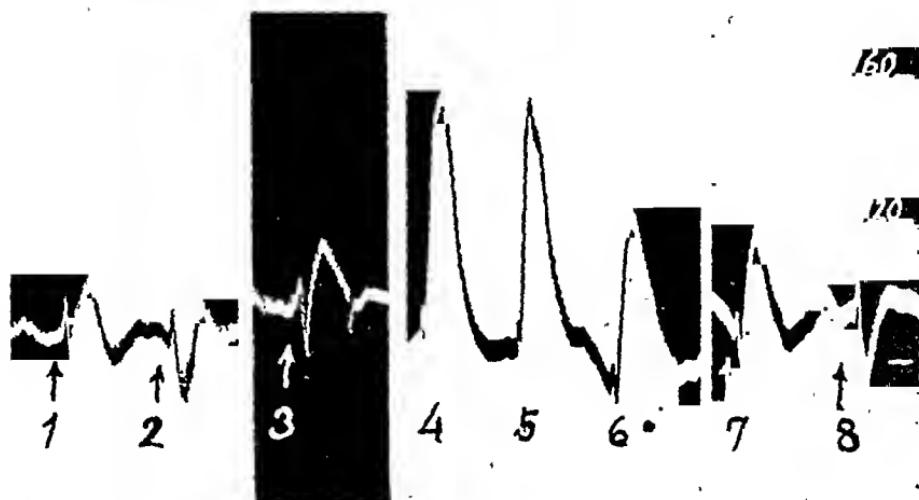


Fig. 2. Blood pressure, cat, chloralose.

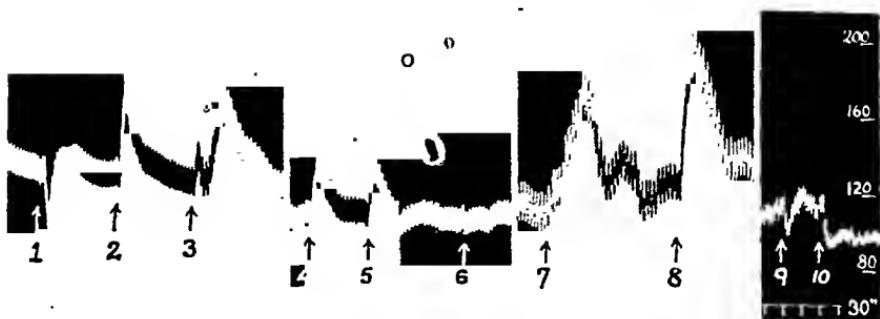
1. 0.2 lipid ether extract of fresh cattle spleen.
2. 0.2 lipid ether extract of spleen stored at room temp. for 5 hours.
3. 0.2 lipid ether extract of fresh liver.
4. (After 10 mg/kg cocaine hydrochloride i. m.) 0.1 of extract 1.
5. 5 µg adrenaline.
6. 0.1 of extract 2.
7. 0.2 of extract 3.
8. 0.2 lipid ether extract of liver stored at room temp. for 5 hours.

for the test since they mostly reacted with fall in blood pressure only, unless highly purified extracts were used.

A question of primary importance was whether the time of preparation of the organ after the death of the animal had any influence on the yield of pressor activity especially in view of the findings of GRABE, KRAYER and SEELKOFF (1934) on pressor actions in liver extracts. It was found that the most active preparations were obtained when the organ was taken out immediately after the slaughtering and minced in cold acid alcohol or stored for a few hours only in the refrigerator before the extraction. Organs left in room temperature for some hours yielded less pressor activity (Fig. 2).

## 2. Purification of extracts.

A disturbing factor was the depressor action regularly preceding the pressor response. Several attempts to separate the depressor principles from the pressor were made by means of treatment with various organic solvents such as alcohol, acetone and ether. It was noticed that the pressor substance or substances were to some extent soluble in mixtures of alcohol and ether, but, unfortunately, this also applied to the depressor effect. The best results were obtained by shaking the aqueous extract after evaporation of the alcohol with a solution of organ lipids in ether and subsequent shaking of the lipid-ether with



*Fig. 3. Blood pressure, cat, chloralose.*

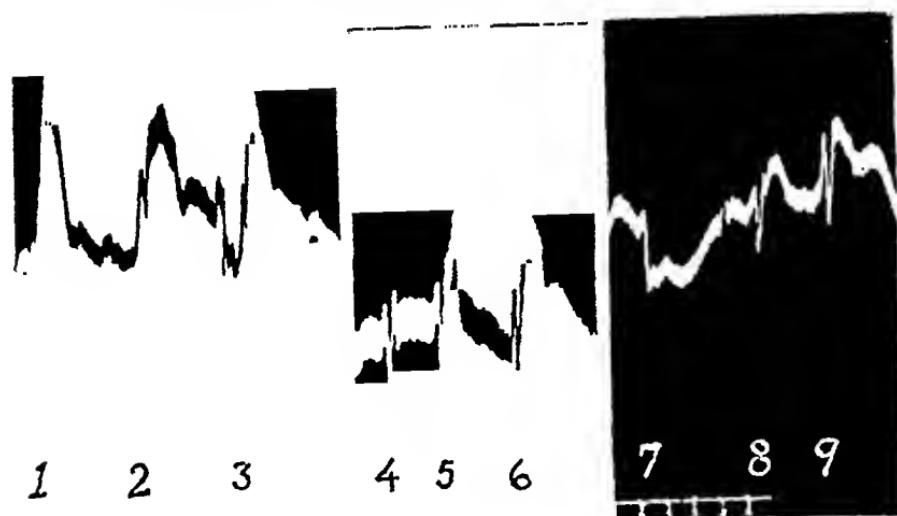
1. Suhlimate precipitate from lipid-ether extract of cattle spleen.
2. Sublimate filtrate.
3. 1  $\mu$ g adrenaline.
4. Same as (2), heated to boiling with N NaOH.
5. Half the dose of (2), untreated.
6. 1  $\mu$ g adrenaline heated to boiling with N NaOH.
7. (After cocaine hydrochloride 10 mg/kg i. m.) 1  $\mu$ g adrenaline.
8. Same as (2).
9. (After ergotamine tartrate 2 mg/kg i. v.) 5 times the dose in (2).
10. 5  $\mu$ g adrenaline.

5—10 % sodium sulphate solution. The sodium sulphate was removed by addition of 3 volumes of alcohol and the filtrate, after evaporation of the alcohol, showed a good pressor effect but only little depressor action (Fig. 7). By this treatment a considerable purification was attained, and a number of biological tests could be performed on the purified extract. These extracts will be termed lipid-ether extracts. After concentration and renewed extraction with lipid-ether there was practically no depressor action left. A further purification was obtained by treatment with  $HgCl_2$ . After complete precipitation with  $HgCl_2$  in alcohol and sodium acetate the filtrate was decomposed with  $H_2S$  and, after removal of the  $HgS$ , concentrated, neutralized with NaOH, evaporated to a small volume and taken up in methanol, filtered and freed from alcohol. Treatment with  $HgCl_2$  removed all of the depressor activity occasionally left in the lipid-ether extracts with the precipitate, from which it could be recovered by decomposition with hydrogen sulphide, as will be seen from Fig. 3. The pressor effect appears to be unchanged as to its general type though some loss occurred and also some changes in stability (see under 4).

### 3. Biological actions of extracts.

#### a. Blood pressure.

The lipid-ether extracts and the  $HgCl_2$ -filtrates were tested on the blood pressure of the cat and the rabbit. The responses on the rabbit in urethane anaesthesia were, on the whole, rather



*Fig. 4.* Blood pressure, cat, chloralose.

1. 2  $\mu$ g dihydroxy-nor-ephedrine (D. N. E.)
2. 2  $\mu$ g adrenaline.
3. 0.02 purified extract of cattle spleen.
4. (After 2 mg ergotoxine per kg) 10  $\mu$ g adrenaline.
5. 10  $\mu$ g D. N. E.
6. 0.1 (3).
- 7.—9. Same as 4—6 after 3 mg ergotoxine per kg.

poor, though a pure action was mostly obtained. On the cat, on the other hand, pure and strong pressor responses were consistently recorded. The pressor action of the extracts was of the same general type as that of adrenaline and was accompanied by an acceleration of the heart. Certain differences in action were noticed, however, which were in some instances so marked that it became highly doubtful whether the active substance was identical with adrenaline, in spite of other observations indicating a near relationship to this substance. Thus, in many animals, adrenaline caused a 'step' (Fig. 3) or a depressor notch whereas an action of this type was never observed with the spleen extracts. Further, the rise in blood pressure usually occurred quicker with the latter as seen from the figure. Usually the increase in heart frequency was more marked with the extracts than with adrenaline.

*Effect of cocaine.* The enhancement of the adrenaline action after a small dose of cocaine, as discovered by FRÖHLICH and LOEWI (1910), also applied to the pressor action of the extracts. (Fig. 1, 2, 3) This strongly indicated an adrenaline-like body, since the cocaine effect appears only with certain dioxyphenol

derivatives. The increase in action was not always parallel in extracts and with adrenaline, but varied in different extracts, according to the degree of purification and previous treatment. Thus the pressor activity left after treating the primary extracts with  $\frac{1}{10}$  of the volume of 10 N NaOH for 10 minutes at 100° C. was relatively less influenced by cocaine (Fig. 1) than the untreated extract.

*Effect of ergotamine.* The probable relationship between the unknown pressor substance and sympathomimetic compounds of the adrenaline type made it desirable to test the action after ergotamine. A dose sufficient to annul or reverse the pressor action of adrenaline (2 mg/kg) strongly decreased the action of purified preparations, but seldom abolished the pressor action completely (Fig. 3 and 4).

The obvious difference in action between adrenaline and spleen extracts after ergotamine is of interest since it is known from the investigation on sympathomimetic amines of BARGER and DALE (1910) that different amines behave differently in this respect. Thus the methylamino-bases (such as adrenaline) after a suitable dose of ergotamine produced a pure fall in pressure on the spinal cat whereas the corresponding amino-base caused a slight rise. On other occasions the action of nor-adrenaline may be wholly inhibited or even slightly reversed by ergotamine though never to the same extent as adrenaline (STEHLE and ELLSWORTH, 1937).

It was therefore considered that the active pressor extracts contained a pressor principle of a type differing from adrenaline and more resembling the type of catechol bases represented by nor-adrenaline. Catechol-ethanol-amine not being available, a number of comparative tests were made with a similar substance, dl 3 : 4-dihydroxy-nor-ephedrine (D. N. E.) prepared by the I. G. Farbenindustrie and kindly put at my disposal by prof. G. LILJESTRAND. This compound was not included in the series investigated by BARGER and DALE but has been studied by SCHAUMANN (1931). The tests with D. N. E. revealed the same type of action as the amino-bases in BARGER and DALE's experiments, and apparently D. N. E. resembles nor-adrenaline to a high extent, quantitatively as well as qualitatively. After a dose of ergotamine sufficient to reverse the action of adrenaline both the extract and the D. N. E. retained a slight pressor effect in doses equi-pressor before ergotamine (Fig. 4).

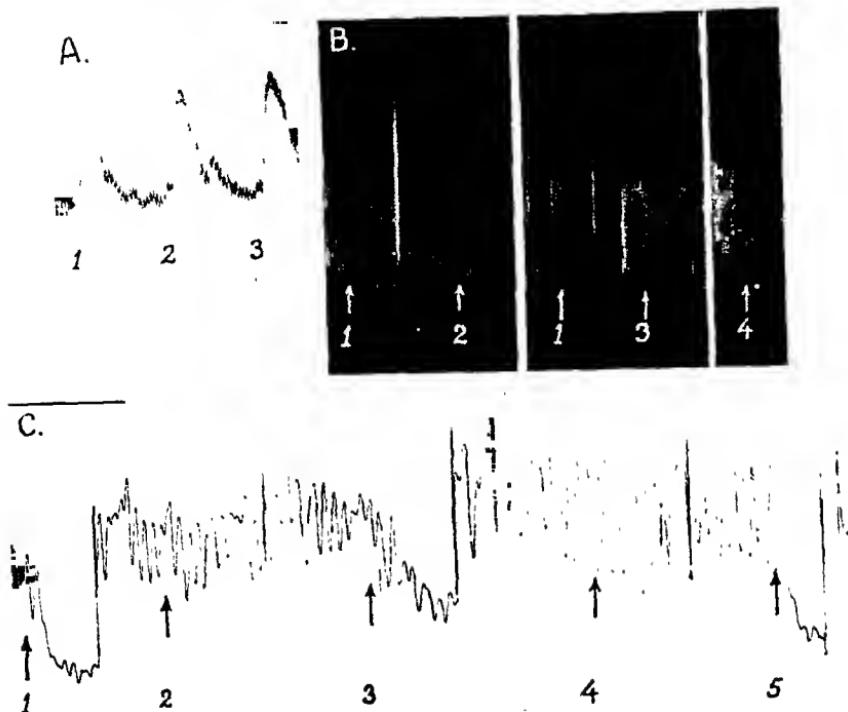


Fig. 5.

- A. Blood pressure, cat, chloralose.
  - 1. 2 µg adrenaline.
  - 2. 0.03 purified spleen extract.
  - 3. 3 µg dihydroxy-nor-ephedrine (D. N. E.).
- B. Isolated rabbit's jejunum.
  - 1. 1 µg adrenaline.
  - 2. 0.01 purified spleen extract.
  - 3. 1 µg D. N. E.
  - 4. 0.5 µg adrenaline.
- C. Isolated non-pregnant cat's uterus.
  - 1. 0.5 µg adrenaline.
  - 2. 0.02 purified spleen extract.
  - 3. 0.05 purified spleen extract.
  - 4. 1 µg D. N. E.
  - 5. 3 µg D. N. E.

If the difference in action of the spleen pressor substance and adrenaline after ergotamine can be interpreted as a weaker inhibitory action on the blood vessels of the former, one should expect the extracts to differ in their action from adrenaline also on other test organs such as intestine or uterus. Equipressor doses of adrenaline and purified extracts of spleen were accordingly tested on these organs.

*b. Effect on intestine.*

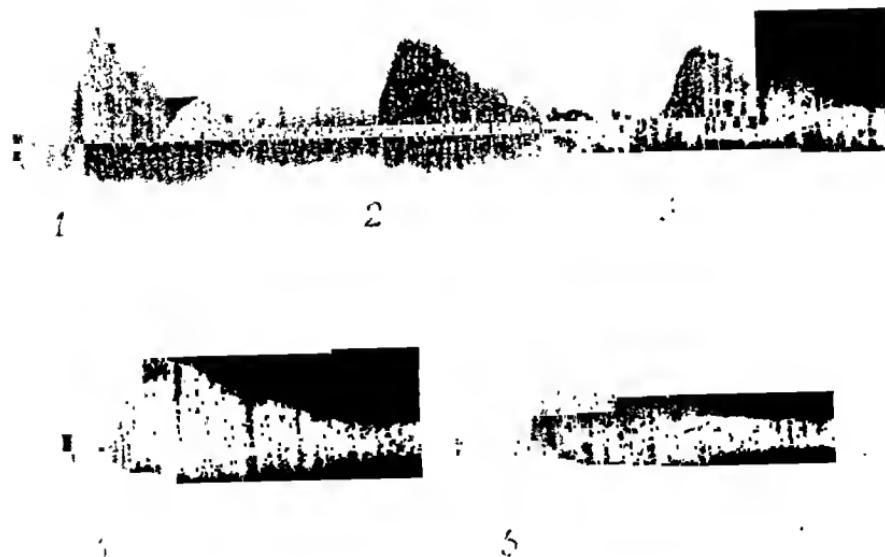
The tests were made on the isolated rabbit's jejunum in Tyrode's solution. A comparison of adrenaline and purified extracts showed also on this preparation a marked difference, the pressor extracts causing a smaller inhibitory effect like that of D. N. E. (Fig. 5). Less purified extracts with depressor action were as a rule strongly stimulating. On this account adrenaline was added to the purified extract which then produced the usual inhibition, showing that an effect of this kind was not masked by the presence of small amounts of stimulating substances left in the extract.

*c. Effect of cat's uterus.*

Further evidence of a real difference between adrenaline and the active pressor substance in the spleen extracts was obtained by testing the active preparations on the virgin or non-pregnant cat's uterus. This organ reacts to adrenaline with a pure inhibition already in small doses, whereas the non-methylated sympathomimetic amines are definitely less active (BARGER and DALE, 1910). The result of the tests was quite decisive, showing the usual fall in tone after adrenaline, whereas an equipressor dose of the pressor extract caused no action or a slight effect only. With higher doses, again, inhibition ensued which was in good accord with the action of D. N. E. as shown in Fig. 5. In extracts purified in different ways varying degrees of inhibitory action have been observed. Thus of two extracts, in doses having the same effect on the blood pressure, one being a simple lipid ether extract and the other further purified by sublimate treatment, the first one caused a slight inhibition of the uterus, the second no change at all, and a dose of adrenaline equipressor with the doses of extracts, caused the usual strong inhibition. It can thus be stated with a fair degree of safety that the spleen extracts contain a substance behaving much like the amino-bases in BARGER and DALE's experiments.

*d. Rabbit's uterus.*

On the rabbit's uterus the action of spleen extracts like those of adrenaline and D. N. E. was a purely stimulating one. A comparison of equipressor doses revealed, however, that the action of the spleen extracts was some 5 times smaller than of adrena-



*Fig. 6. Isolated cat's heart, Langendorff preparation.*

1. 1  $\mu$ g 3:4-dihydroxy-nor-ephedrine (D. N. E.).
2. 0.012 purified spleen extract.
3. Equipressor dose of (2) purified over amy1 alcohol.
4. Twice the dose in (3).
5. 1  $\mu$ g adrenaline.

line, whereas that of D. N. E. was some 20 times weaker. Even on this test object, consequently, the spleen extracts showed a distinct difference against adrenaline.

#### e. Isolated heart.

Purified extracts of spleen were tested on the isolated perfused heart of the cat and the rabbit according to LANGENDORFF and compared with adrenaline and D. N. E. The latter substances both stimulated the heart to about the same extent in the same doses as stated by SCHAUMANN (1931) and the purified spleen extracts had a very similar effect in equipressor doses (Fig. 6).

#### f. Effect on the pupil width.

Injections were made either intravenously or intra-arterially through the common carotid in central direction. A comparison of the pupil-dilating effect of active extracts and adrenaline

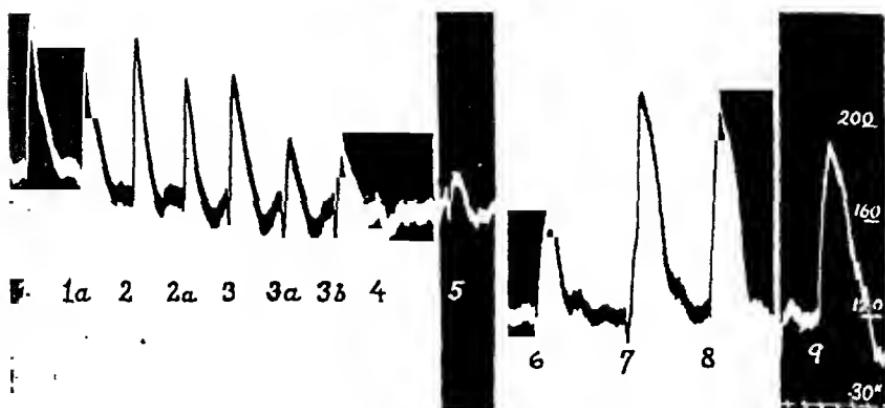


Fig. 7. Blood pressure, cat, chloralose.

1. 0.5  $\mu$ g adrenaline.
- 1a. Same heated 5 min. at 100° C. with N H<sub>2</sub>SO<sub>4</sub>.
2. 0.5  $\mu$ g 3:4-dihydroxy-nor-ephedrine (D. N. E.).
- 2a. Same, treated as in (1a).
3. 0.1 purified spleen extract.
- 3a. Same, treated as in (1a).
- 3b. Same, heated to boiling with N NaOH.
4. 0.5  $\mu$ g D. N. E. treated as in (3b).
5. 0.1 (3) heated 5 min. at 100° C with N NaOH.
6. (After cocaine hydrochloride 10 mg/kg i. m.) 0.1 (3b).
7. 0.1 (3).
8. 0.5  $\mu$ g D. N. E.
9. 0.5  $\mu$ g adrenaline.

showed a very marked difference in action; adrenaline widened the pupil much more than equipressor amounts of extracts. The possibility that impurities with constrictor action were operating in the extracts could be ruled out by the fact that addition of an amount of adrenaline to the extract, less than half of the equipressor dose, still produced a definitely stronger dilation than the extract. Still a slight widening was regularly observed with the purified extracts, indicating a sympathomimetic action, yet different from that of adrenaline.

#### 4. Stability, solubility.

The active extracts lost most of their biological activity on heating to 100° in normal alkaline solution for 5 minutes. When just heated to boiling after addition of one tenth of the volume 10 N NaOH to the slightly acid lipid-ether extract, about 50 % of the activity was still left whereas adrenaline or D. N. E. was completely destroyed. Further purified extracts lost, however,

as much of their activity as equipressor doses of adrenaline or D. N. E. Whether the lipid-ether extracts still contained buffering substances in sufficient amount to prevent a full effect of the added alkali or whether contaminating substances with protecting action are responsible for the difference cannot be decided.

In the primary extracts (before lipid-ether extraction) a certain fraction of the pressor activity was left even after 10 minutes' heating at 100° with one tenth of the volume of 10 N NaOH, indicating a very stable pressor substance (Fig. 1). As seen in the figure the remaining effect was not enhanced by cocaine in the same proportion as the untreated extract indicating the presence in the primary extracts of a substance resembling tyramine in its action, which is depressed by cocaine as shown by TAINTER and CHANG (1933), and also resists 10 minutes' heating at 100° in normal acid or alkaline solution.

On the other hand there is a certain amount of activity left, still causing a pressor action after cocaine (Fig. 1 (6)). This remarkable stability is lost as a result of further purification and may be related to the presence of stabilizing agents, as in the experiments by HEARD and RAPER (1933) on perfusion of the adrenal gland.

Heating with normal sulphuric acid likewise diminished the action, though less readily. The following table illustrates the inactivation by treatment with alkali and acid, denoting per cent activity left.

The purified extracts are easily inactivated by treatment with oxidizing agents such as iodine or potassium permanganate which is in accord with the other evidence for catechol bases.

	Lipid-ether extract	Filtrate from HgCl <sub>2</sub> -precip.	D. N.E.	Adrenaline	Tyramine
<i>A. Normal alkali</i>					
heated to boiling in 10 secs. ....	>50	<20	<20	<20	100
5 mins. 100° ....	20-50	0	0	0	100
10 " 100° ....	20-30	0	0	0	100
<i>B. Normal acid</i>					
5 mins. 100° ....	50-100	50	70	70	100
10 " 100° ....	50-100	<20	50	50	100

The results regarding solubility are probably less significant since they apply to extracts, containing comparatively large amounts of impurities in spite of the relative high purification as

to the biological effect. The solubility in alcohol showed great variations according to the degree of purification of the extracts. The solubility in ether or chloroform was negligible. In conformity with this it has not been possible to obtain but a trifling yield on continuous fluid extraction with ether at pH varying from 3 to 8.

As already stated above the active substance dissolves to some extent in an ether solution of organ lipids or pure lecithine. When other ether soluble vehicles such as triglycerides, oleic acid, paraffine oil, or bee-wax were used the yield was much less, being highest for wax. There seems to be a definite quantity of the pressor substance carried by a given amount of lipids in ether solution, since the yields have been much the same in each of 20 successive extractions. It has also been possible to show that adrenaline, added to the extract, is extracted in a similar proportion as the inherent pressor activity.

##### 5. Colour reactions.

Many of the almost colourless purified extracts contained amounts of pressor activity corresponding to some 50—100  $\mu\text{g}$  adrenaline per ml. Assuming a similar chemical composition and an activity not greatly exceeding that of adrenaline per unit of weight it should be possible to obtain some of the typical colour reactions for this substance.

A rather sensitive colour reaction, but of little specificity, is the Folin-Cannon-Denis test with tungstic acid, which was always positive but usually much stronger than the reaction with an equipressor amount of adrenaline probably owing to impurities.

The simple  $\text{FeCl}_3$  test, on the other hand, gave a greenish tint of the same order of strength as adrenaline for equipressor amounts in purified extracts, changing to red after alkalization. This reaction, indicating a catechol nucleus, often proved useful in estimating the approximate strength of the extracts as to the pressor activity.

When the Vulpian iodine reaction was employed according to the method described by EULER (1933), it was not possible, however, to obtain any red colour, though addition of adrenaline in amounts less than half of the total pressor activity caused a definite reaction. It was noted, that the extracts obtained a slight

straw yellow colour, sometimes changing to greyish, like that of dioxiphenyl-ethyl-amine, though this gave an initial strong red colour with iodine.

From the colour test experiments it may be inferred that the active substance, probably is a catechol compound of the same order of activity as adrenaline. These findings seem to rule out a number of sympathomimetic amines, such as hydroxytyramine, which is some 50 times weaker in its pressor effect than adrenaline, and many others. Among the known catechol derivatives only dihydroxy-phenyl-ethanol-amine and its  $\beta$ -methyl-substituted derivative seem to come into question on account of their activity and biological actions.

Of the physical methods for detecting adrenaline that of GADDUM and SCHILD (1934), using the green fluorescence of adrenaline in alkaline medium in the presence of oxygen, has the advantages of being very sensitive and quite specific. The reaction was carried out with equipressor concentrations of purified spleen extract, adrenaline and dihydroxy-nor-ephedrine, both the latter in a strength of 1: 100,000. After appropriate dilution to compare biologically with the adrenaline solution, the purified spleen extract was almost colourless and showed only a very weak greenish fluorescence of its own. After addition of alkali the adrenaline solution showed a strong green fluorescence, whereas no visible change was noted in the other solutions. Addition of adrenaline to the spleen extract, corresponding to  $1/_{10}$  of the pressor activity (1  $\mu$ g in 1 cc) still produced a distinct reaction, showing that if any adrenaline was present in the extract the amount was certainly less than the added amount. These results give a definite proof that the active substance is different from adrenaline as already indicated by the biological tests. At the same time they give further indirect support to the assumption that the active substance is identical with or closely related to catechol-ethanol-amine, which gives a very weak fluorescence reaction, about 2 p. c. of that of adrenaline, as found by GADDUM and SCHILD.

From these experiments it appears that the active substance in the purified spleen extracts shows a much closer agreement with the action of 3:4-dihydroxy-nor-ephedrine than with that of adrenaline. Unfortunately it has not yet been possible to compare the actions of the extracts directly with 3:4-dihydroxy-ethanol-amine but it is evident from the experimental data in the literature concerning its biological actions (BARGER and DALE, 1910, GREER,

PINKSTON, BAXTER and BRANNON, 1937) that a close relationship should exist between this substance and the chief active principle in the extracts.

### Comments.

In a preliminary report (EULER, 1945) it has been shown that considerable amounts of a sympathomimetic 'pressor' substance may be found in a number of fresh organ extracts. From the results put down in the present paper it emerges that the active substance in spleen extracts, which have been subjected to a closer study, is related to, but different from adrenaline.

The careful analysis of the action of sympathomimetic substances on various biological test objects in the classical study of BARGER and DALE (1910) has brought to light certain important differences between the action of the methylamino-bases of the catechol group, such as adrenaline, on the one hand, and the amino-bases — and to a lesser degree the ethyl- and propylamino-bases — on the other. It is possible, and even probable, that the spleen extracts contain more than one active substance belonging to the oxyphenyl- or catechol groups but it seems safe to assume that at least an important fraction of the activity is due to a substance having the characteristic features of a catechol amino-base. Direct comparison has shown an extensive agreement with the isomer of adrenaline, 3 : 4-dihydroxy-nor-ephedrine which has been used in lack of the 3 : 4-dihydroxy-ethanol-amine, the non-methylated adrenaline or nor-adrenaline.

The significance of these findings is closely connected with the fact that the biological effects of sympathetic stimulation only partly agrees with that of adrenaline and that a number of stimulation effects show a much closer agreement with the actions of the amino-bases of the catechol group as pointed out by BARGER and DALE. The following may be quoted from their paper:

"The conception of sympathetic nerve-impulses as acting by the liberation of adrenaline seems to us unsatisfactory for another reason. It involves the assumption of a stricter parallelism between the two actions than actually exists. Adrenine has, in common with the other methylamino-bases of the catechol group, the property of exaggerating inhibitor as compared with motor effects. The inhibitor effects of these methylamino-bases are relatively prominent not only as compared with those of homo-

logous bases, in particular the aminobases, but also in comparison with those of sympathetic nerves. All the sympathetic effects which are weakly or doubtfully reproduced by adrenine are motor effect — *e. g.* pilo-motor action, or contraction of the trigone of the cat's bladder. On the other hand certain inhibitor effects, such as inhibition of the fundus of the cat's bladder, or of the non-pregnant uterus of the same animal, are more easily and completely produced by adrenine than by nerve-stimulation. Similarly certain normally motor effects of adrenine are reversed by smaller doses of ergotoxine than are needed for the reversal of the corresponding motor effects of stimulating sympathetic nerves. In these respects the action of some of the other bases, particularly of the amino- and ethylamino-bases of the catechol group, corresponds more closely with that of sympathetic nerves than does that of adrenine. To suppose that such bases and sympathetic nerve-impulses alike owe their action to the liberation of adrenine seems to us to create additional difficulties of conception."

BARGER and DALE's inferences regarding the nature of a possible mediator substance involved in sympathetic stimulation (adrenergic fibres) has received strong indirect support from the experimental work of CANNON and his associates, BACQ and ROSENBLUETH, on sympathin. They showed that stimulation of certain sympathetic nerves produced remote actions, proving the liberation of a kind of substance, which was obviously related to but, in certain respects, different from adrenaline. The latter substance was, however, regarded as the primary mediator. By assuming the formation of special cell reaction products, sympathin E and I, CANNON and ROSENBLUETH (1933) made an attempt to interpret their observations. Neither of the postulated sympathins has, however, hitherto been isolated or even prepared in such a way that their properties could be put to a decisive test.

The similarity between the excitatory effects of sympathetic stimulation and of non-methylated adrenaline, as originally shown in BARGER and DALE's experiments, led BACQ (1934) to express the thought that the latter was the excitatory mediating substance and adrenaline the mediator where inhibitory actions were prevailing. This view has been shared by STEHLE and ELLSWORTH (1937). Similarly GREER, PINKSTON, BAXTER and BRANNON (1936) point out, as a result of their experiments, that stimulation of the hepatic nerves causes effects which are in better accord with the action of nor-adrenaline than with adrenaline. The pres-

ent findings have given direct proof of the occurrence in spleen extracts of a substance with similar properties as those characteristic of sympathin E though it should be noted that it also has a weak inhibitory action. Since the FeCl<sub>3</sub> catechol reactions for equipressor solutions of adrenaline and spleen extract are similar in strength it is inferred that the active substance must be of the same order of activity as adrenaline. This gives some indication as to the exact nature of the unknown substance, since a similar quantitative relation so far is known to exist only for adrenaline, nor-adrenaline and dihydroxy-nor-ephedrine. Assuming that the active substance in spleen extracts is also the substance liberated on sympathetic stimulation in this organ the probability of the identity of the latter with nor-adrenaline is greatly increased

### Summary.

1. Extracts of fresh cattle spleen possess a pressor activity equivalent to some 10 µg adrenaline per g of tissue.
2. The purified substance increases the heart rate and raises the blood pressure of the cat in chloralose anaesthesia.
3. The pressor action is enhanced by cocaine.
4. Ergotamine in doses which annul or reverse the pressor action of adrenaline is less active in depressing the action of purified spleen extracts, which in this respect resembles certain catechol amino-bases, such as nor-adrenaline or 3:4-dihydroxy-nor-ephedrine (D. N. E.).
5. Adrenaline inhibits the isolated rabbit's intestine and the non-pregnant cat's uterus more powerfully than equipressor doses of spleen extracts or D. N. E.
6. Purified spleen extracts, like D. N. E., are less active in stimulating the rabbit's uterus than equipressor doses of adrenaline.
7. Purified spleen extracts and D. N. E. have a weaker pupil dilating action than equipressor doses of adrenaline.
8. Purified spleen extracts stimulate the isolated heart in much the same way as equipressor doses of adrenaline and D. N. E.
9. Purified spleen extracts and D. N. E. do not give the fluorescence reaction characteristic of adrenaline in equipressor concentrations.

10. Purified spleen extracts and D. N. E. give the FeCl<sub>3</sub> colour reaction to about the same strength as equipressor concentrations of adrenaline.

11. The biological tests, colour and fluorescence reactions of purified spleen extracts thus bear a good resemblance to those of nor-adrenaline or D. N. E. and differ from those of adrenaline.

12. The similarity between the action of the purified spleen extracts and the postulated sympathin E on the one hand and nor-adrenaline or D. N. E. on the other is pointed out.

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## Protein Metabolism of Tissue Cells in vitro. 4.

### The Properties of Malt Extracts and of Glutathione as Accessory Growth Substances.

By

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In continuation of our previous investigations on substances interfering with the protein metabolism of tissue cells in vitro, (FISCHER 1941, 1942), we have recently studied the accessory growth substances contained in yeast and barley malt (ASTRUP, FISCHER and VOLKERT 1945, ASTRUP and FISCHER 1945). In this paper we are presenting further investigations on the properties of the accessory growth substances in barley malt together with investigations on glutathione. The technique is the same as described in previous papers, dialyzed media being used.

### Experimental.

#### 1. Crude Extract. Method II.

For the easy preparation of larger amounts of malt extract the method described previously (ASTRUP and FISCHER 1945), was found inconvenient. This method will be referred to as *Method I* for the preparation of crude extract. The following method II, in which the time consuming centrifugation of the mixture of water and malt was avoided, was therefore used.

200 g of pulverized barley malt is added to 500 ml water at 35—40° and stirred at this temperature for one hour. 1.6 liter of ethanol (96 per cent) is added, and after standing for a couple of days, the mixture is filtered on a Büchner funnel. The filtrate is evaporated in

vacuo on a waterbath ( $< 50^\circ$ ) to about 300 ml and the lipoids removed by filtration. After dilution to the original volume and neutralization, it is sterilized and tested on the tissue cultures in the usual manner.

This malt extract contains about the usual amount of nitrogen (0.8—1.00 mg per ml). By the addition of ethanol considerable amounts of lipoids and coloured matter are extracted from the malt, but these are subsequently removed by the evaporation and filtration of the aqueous solution.

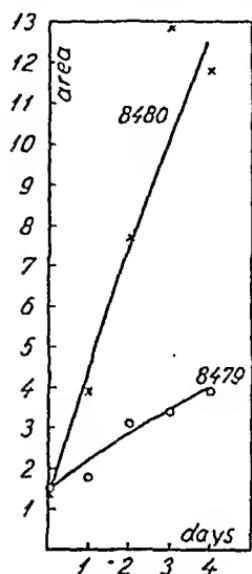


Fig. 1. Activity of crude extract, Method II (V-243.1). Culture no. 8480, 0.1 ml of the extract. Culture no. 8479, no addition.

The extract seems just as active as an extract prepared in the usual manner, see fig. 1. In the following experiments the erude extract was as a rule prepared after this method and eventually concentrated further in vacuo to one tenth of the original volume. In this form it could be kept several months in the ice box without deterioration.

## 2. Glutathione.

As glutathione is a universal constituent of living eells and is an activator for certain enzymatic reaetions in the organism, f. i. proteolytic processes, it might be of interest to test this substance. In previous papers (FISCHER and ASTRUP 1942, ASTRUP FISCHER and VOLKERT 1945), glutathione was investigated after autoelaving, as it

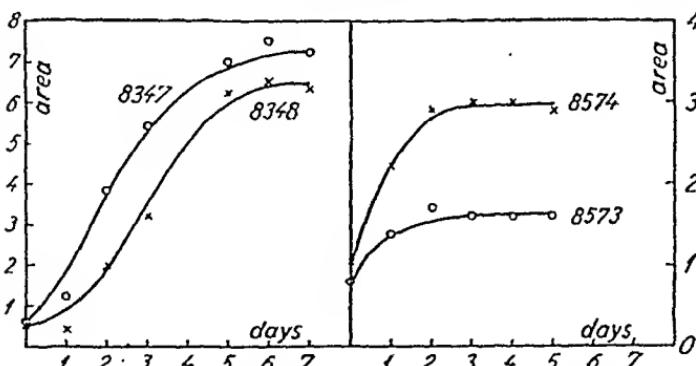


Fig. 2. Action of glutathione. Culture no. 8347, 0.2 ml of purified malt extract (V-231.1, corresponding to V-217.6 by FISCHER and ASTRUP (1945)). Culture no. 8348, 0.2 ml of 0.2 per cent glutathione (V-241). Culture no. 8573, no addition. Culture no. 8574, 0.2 ml 0.1 per cent glutathione (V-248.1).



Fig. 3. Photographs of living heart fibroblasts in Carrel flasks. Magnif. 40 $\times$ . No. 8949, Mother liquor from malt extract treated with cadmium lactate after Binet and Weller (V-250,1). No. 8950, glutathione (V-252,3). No. 8927, oxydized glutathione (V-259).

should be compared with fractions active after such treatment. We now tried the substance after sterile filtration. Glutathione was prepared from bakers yeast after the methods of PIRIE (1930) or SCHROEDER, COLLIER and WOODWARD (1939). Solutions were prepared in physiological saline, almost neutralized and sterile filtered through sintered glass filters.

Glutathione, when used in not too low concentrations, shows some activity on the tissue cultures. Comparatively large areas of growth may be obtained, but the cells are not of normal appearance and very granulated; the cells disintegrate in the course of 4—6 days. Its action is rather similar to the action of cystine, though larger areas may be obtained. Some results are shown in Fig. 2 and 3. Oxidized glutathione shows the same activity. Autoclaving does not completely destroy it.

The experiments with glutathione show that this substance, although active to some extent, is not responsible for the full effect of the malt extracts. Another question is, to what extent it contributes to this effect, or if other active substances are the sole cause. This may be disclosed by preparing glutathione-free fractions of the malt extract. The content of glutathione in different extracts was therefore determined after the colorimetric method of FUJITA and NUMATA (1939).

### 3. Glutathione in the Active Extracts.

Yeast is a rich source of glutathione, and the active yeast extracts previously investigated (ASTRUP, FISCHER and VOLKERT 1945) proved to contain amounts of this substance, sufficient to allow its isolation over the cuprous merkaptide compound. Malt extract also contains glutathione, but in considerably smaller amounts. Prepared after *Method I* it contained about 0.14 mg total glutathione per ml; of this 0.08 mg per ml was in the reduced form. These amounts are far smaller than the concentrations of glutathione necessary in pure solutions for producing growth of any extent (about 2 mg per ml), and do not indicate any significance of glutathione for the growth promoting properties of malt extracts.

It was attempted to remove the glutathione from the solutions by means of Cu<sub>2</sub>O (HOPKINS 1929) or using the cadmium method of BINET and WELLER (1935), but the resulting solutions still contained small amounts of glutathione.

V-245,1 is prepared by Method I and purified by one precipitation with ethanol at basic reaction. It contains 0.54 mg N per ml and 0.14 mg total glutathione per ml. After concentration to  $\frac{1}{4}$  volume a small precipitate separates after treatment with  $Cu_2O$  in the usual manner. Copper is removed from the precipitate, and it is tested on tissue cultures in four times the concentration of the original extract. It contains now 0.11 mg total glutathione per ml and 0.06 mg N per ml (V-245,2), but shows no effect.

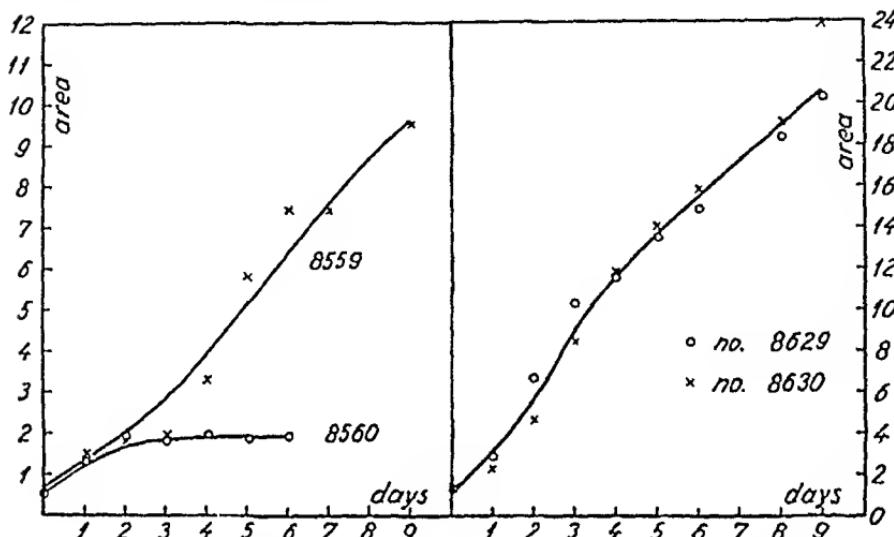


Fig. 4. Culture no. 8559, original malt extract, V-245,1 (0.1 ml). Culture no. 8560,  $Cu_2O$  precipitate, V-245,2. (0.1 ml). Culture no. 8629, mother liquor from  $Cu_2O$  precipitation, V-245,3 (0.2 ml). Culture no. 8630 original malt extract, V-245,1 (0.2 ml).

$H_2SO_4$  is removed from the centrifugate from the  $Cu_2O$ -treatment by means of  $Ba(OH)_2$ , and diluted to the original volume (V-245,3). It contains 0.06 mg total glutathione and 0.19 mg N per ml and seems just as active as the original solution V-245,1, see fig. 4. Similar results were obtained after treatment with cadmium (V-250) and seem to exclude any major significance of glutathione for the growth promoting properties of malt extracts.

Some malt extracts were treated with  $H_2S$  in order to convert all glutathione present into the reduced form to facilitate the complete precipitation of it. While this object was not reached, it was found, that during the treatment with  $H_2S$  a considerable amount of a yellow precipitate separated. This treatment as a rule did not reduce the activity, but considerable amounts of nitrogen were removed from the solution.

Neither treatment with  $H_2$  or  $CO_2$  influences the activity nor

produces a precipitate. At neutral or alkaline reaction no precipitate is formed by means of  $H_2S$ . The nature of the precipitate is unknown and was not investigated.

#### *4. The Sugar Content of the Malt Extract.*

Many of the difficulties met with in the purification of the extracts found to be due to the considerable amounts of sugar contained in the solution. The sugar content was therefore followed during the operations by means of the method of HAGEDORN and JENSEN. Most of the sugar present in the extracts must be assumed to be maltose, of which one mol. (342) requires about fifty per cent more oxygen than one mol. of glucose (180), see SOBOTKA and REINER (1930) and MICHEEL (1939). This must be taken into consideration, when making use of the amounts of sugar generally determined in the Hagedorn and Jensen method, and which are stated without conversion in the following investigations. Also glutathione influences the sugar determination (HERBERT, BOURNE and GROEN, 1930), but this is of no importance in this connection, due to the small amounts of glutathione present.

A crude extract II (V-273,1) showed a content of 26.1 mg sugar per ml solution before the alkaline treatment and 19.9 mg per ml after. After treatment with  $H_2S$  (V-273,2) it contained 16.5 mg sugar per ml. None of these operations thus removed any considerable part of the sugar content.

A concentrated malt extract (V-275) after alkaline precipitation and treatment with  $H_2S$  contained 21.8 mg sugar per ml (diluted to the original volume) (V-275,2). After precipitation with acetic acid and ethyl alcohol, see ASTRUP and FISCHER (1945), the active solution contained 10.0 mg sugar per ml (V-275,3). This treatment thus removed about half of the amount of sugar present.

Several attempts were made to remove by simple chemical means the large amounts of sugar still present but without much success. Fractionated precipitation with methyl alcohol was tried. A concentrate made acid to congo paper with diluted HCl was soluble in methyl alcohol. After the addition of absolute alcohol, an inactive precipitate containing a considerable amount of sugar appeared, but this treatment seemed not more advantageous than the precipitation with acetic acid and ethyl alcohol, (V-273, V-274).

In previous investigations on the accessory growth substances present in yeast (ASTRUP, FISCHER and VOLKERT 1945), we found, that treatment with active carbon removed a considerable part of nitrogen containing impurities and only small amounts of the active substances. The partially purified (acetic acid) malt extracts were treated with active carbon in order to find means for absorbing the active substances,

but also in this case they were to a large extent left behind in the solution. Only minor amounts of sugar and nitrogen were removed at neutral reaction by one or two treatments with active carbon, and the resulting solution was almost as active as the original solution (V-273,7, V-276,5). Repeated treatment (five times) removed however the active substance and about half the amount of sugar and nitrogen (V-273,9). In acetic acid solution three successive treatments with active carbon removed most of the active substances, only small amounts of sugar and considerable amounts of nitrogen (V-275,5). But it was not found possible to recover the active substances adsorbed on the carbon. Treatment with 33 and 50 per cent acetic acid, NaOH to reaction on phenolphthalein and diluted NH<sub>4</sub>OH yielded no results. Treatment of less purified solutions, containing more sugar, with active carbon resulted in still poorer adsorption of the active substances (V-277,2).

### Discussion.

Glutathione was found by BAKER (1928) to increase the growth of fibroblasts in vitro. HUEPER and RUSSELL (1933) stated that concentrated embryo extracts as used in tissue culture contained 40 mg glutathione per 100 ml and found cysteine and glutathione to increase cellular growth. In plasma glutathione disappears rapidly. The growth promoting substances extracted from sprouting maize were assumed not to be glutathione, even if SH-groups eventually played a rôle (PAULMANN 1939).

According to our investigations glutathione has definite effect on dialyzed media, but is not able to replace an extract of barley malt. It is also generally accepted that glutathione rapidly disappears in plasma (OBERST 1935, WOODWARD 1939), and that the content of glutathione in blood is exclusively due to the glutathione contained in the red blood cells, cf. USSING (1943). It may therefore also be assumed, that the accessory growth properties of native plasma or plasma dialysates are not due to a content of glutathione, but according to later experiments by NUMATA (1940) plasma still contains small amounts of glutathione in the oxidized form, about 7 mg per 100 ml. This is of the same order as found by us in the active malt extracts, but is considerably smaller than the amounts found necessary to produce any significant growth of the tissue cultures on the dialyzed media.

As the malt extracts do not contain any considerable amounts of glutathione, and preparations containing still lesser amounts are fully active on the tissue cultures, this indicates that glutathione is of only minor importance, if any, for the accessory

growth properties of malt extracts. By the methods investigated, it was not possible quantitatively to remove glutathione from the solutions; probably the large content of sugar was responsible for this.

Other known substances present in living cells could probably be identical with the substances sought for, either alone or in conjunction with glutathione. We have tried in this manner ascorbic acid and pantothenic acid, but without any success. A solution of 0.2 per cent ascorbic acid (after almost neutralization and sterile filtration) was inactive with or without the addition of glutathione (V-258). This was also the case with pantothenic acid (0.2 per cent solution, sample from "Hoffmann-La Roche", V-238, V-294), although experiments by PRATT and WILLIAMS (1939) indicate a probable action on the respiration of tissues *in vitro*. Several of the properties of our substances seem similar to the properties of pantothenic acid, cf. WILLIAMS, TRUESDAIL, WEINSTOCK, ROHRMANN, LYMAN and McBURNEY (1938). In addition to the chemical difficulties met with in handling such substances, we meet, however, also the obstacle of being without any means for quantitative determination of the amount of active substance present, as the tissue culture method only yield a rough idea about the potency of the preparations. The only result of any reliability is obtained, when the solutions are practically inactive.

Mangano chloride was tried, with and without the addition of glutathione, but was found inactive. p-amino-benzoic acid was inactive and the same was the case with glycocoll.

Some of the properties disclosed in addition to those previously mentioned (ASTRUP and FISCHER 1945), are the following: A relative stability against diluted aqueous and alcoholic alkali in the cold, but not when heated; a higher solubility in methyl alcohol than in ethyl alcohol. The active substance is not adsorbed on barium sulfate or franconite and only with difficulty on active carbon. Treatment with  $H_2S$  does not destroy the substances. By fractionation it follows as a rule the sugar fraction, and it was not found possible by simple chemical means to remove the sugar from the solutions without interfering with the active substances. Later it was found, that a fermentation with yeast removed nearly all sugar without introducing new impurities nor affecting the active substances, and the purification based on this treatment is now under investigation.

Concerning the function of the active substances, it may be mentioned that in reality we do not know in which processes of the living cells they interfere. We have assumed, that it is a question of the protein metabolism, and that this is concerned seems unquestionable, but we do not know, if the substances interfere directly with these reactions, or if they are necessary for the living cells in other respects, a normal protein metabolism being only a sign of normally functioning cells.

### Summary.

1. It is found, that glutathione is an accessory growth substance for tissue cultures, but that it does not suffice to give the cultures normal growth and appearance.
2. The malt extracts previously investigated contain small amounts of glutathione, but this substance is not responsible for the complementary effect of the malt extracts on dialyzed media.
3. Further properties of the active fractions are described.

Most of the analytical determinations in this work were carried out by stud. polyt. KAREN BUHL CHRISTENSEN. For valuable facilities in the preparations our thanks are due to A/S "Ferrosan", Copenhagen, and the barley malt was placed at our disposal by the Carlsberg Breweries.

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## Aerobic Recovery after Anaerobiosis in Rest and Work.

By

ERLING ASMUSSEN.

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The energy required for maintenance and functioning of the organism is ultimately derived from oxydations of the foodstuffs. In a steady state of rest or work the oxygen intake, therefore, is an expression of the energy production. Under certain conditions, however, the energy requirements are greater than the instantaneous oxydations can respond to; the energy then will be liberated by anaerobic processes and an *oxygen debt* is contracted. In subsequent periods of recovery this oxygen debt will be "repaid" as shown by the excess oxygen intake following a period of anaerobic activity. It is the purpose of this paper to investigate the efficiency of this repayment.

The anaerobic conditions studied were partly experimental, viz. produced by blocking of the circulation to the lower extremities, partly those prevalent during the initial stages of physical exercise.

### Methods.

Oxygen uptakes were determined by the DOUGLAS-bag method, the air analyses being done on the KROGH-HALDANE apparatus. Blood lactates were determined after EDWARDS (1938) on finger blood.

A blocking of the circulation to the legs was effected by means of pneumatic cuffs round the thighs as proximally as possible. On sudden release of the pressure in the cuffs a fall in the arterial blood pressure will occur which in turn will be followed by a compensatory increase in circulation rate, emptying of blood depôts etc. and an increase in

oxygen uptake which, however is largely used to oxygenate the venous blood of the blood depôts. (Comp. ASMUSSEN, CHRISTENSEN and NIELSEN (1938, 1939)) In order to avoid this, the subject was placed with the legs in a slightly elevated position (ASMUSSEN, CHRISTENSEN and NIELSEN (1939)) and the pressure in the cuffs was released slowly so that no sudden fall of blood pressure occurred.

The work experiments were performed on a KROGH-bicycle ergometer which for these experiments had been furnished with a reclining chair instead of the ordinary saddle, so that the subject could pass from rest to work and *vice versa* with a minimum of extra work. The chair was adjusted so, that also during exercise the extra work necessary for fixating the body was practically nil, the arms, head and back of the subject resting relaxed on proper supports.

All the experiments were made under standard conditions in the morning. A resting period of 1 hour in the lying or recumbent position preceded each experiment. As subject served E. A., a normal male, 38 years old, height 172 cm, weight 70 kg, well accustomed to and trained in all the experimental procedures.

## Results.

### a. Circulation blocked in rest.

In these experiments the subject was lying on his back with the legs slightly elevated. When the resting oxygen uptake of the subject had been determined in two 5 minutes' periods the cuffs round his thighs were inflated, and one or more determinations of the oxygen uptake were made. In confirmation of earlier experiments (ASMUSSEN, CHRISTENSEN and NIELSEN 1939, b) it was found to be about 20 cc/min lower with the circulation to the legs blocked than in the normal condition. (Mean of 18 experiments:  $19 \pm 1.3$  cc/min). After 5 to 23 minutes the circulation to the legs was restored, and the oxygen taken up during recovery was determined quantitatively until resting values again were found, usually after less than 8 minutes. The oxygen was calculated and plotted against the time of anaerobiosis as shown in fig. 1. In the same figure is shown the magnitude of the oxygen deficit, calculated from the assumption that this is 20 cc/min. It is evident from the figure that when the circulation to the legs has been blocked for more than 5 minutes, the excess oxygen uptake during recovery is greater than the debt contracted during the anaerobic period, and the more so the longer the period of occlusion has been.

Determinations of the blood lactates during recovery showed a slight increase of 4 to 5 mg pCt in the experiments with the

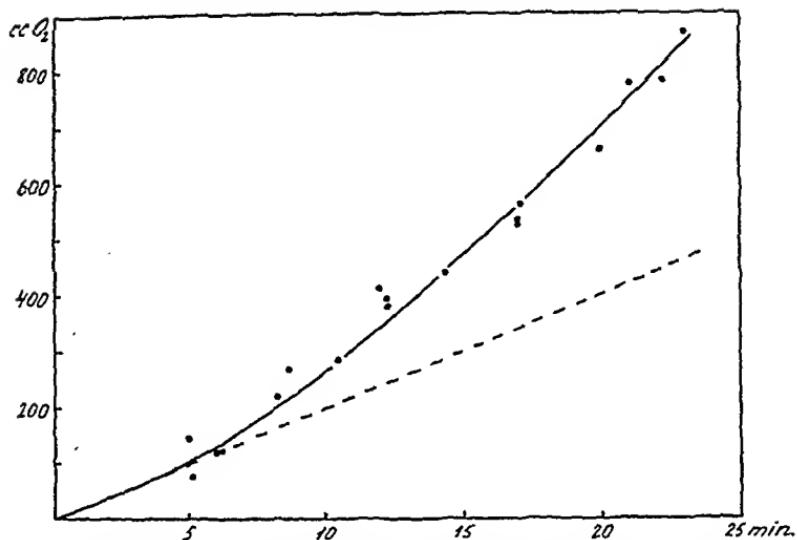


Fig. 1. Subject at rest. *Full line and points:* Excess oxygen taken up after restoration of circulation to the legs. *Dashed line:* Oxygen deficit caused by the occlusion of the circulation to the legs. *Abscissa:* Duration of occlusion in min.

longest time of occlusion, whereas the blood lactates were unaltered in the experiments of shorter duration.

It might be objected against this result, that perhaps the muscles of the thighs have contracted involuntarily during the period of blocking, thus making the oxygen debt greater. Actually the subject was lying completely relaxed, and tests in which an ADRIAN-BRONK needle electrode, connected through an amplifier with a loudspeaker was thrust into the muscles of the leg, made it clear that no muscular movements occurred.

Judging from fig. 1 it must be said that the organism must pay a rather heavy "interest" — up to about 90 pCt — on the "debt" it contracted during the time of occlusion. The ratio *deficit: repayment* is 1 after 5 min but decreases to 0.54 after 23 minutes of blocked circulation.

#### b. Circulation blocked during exercise.

These experiments were performed while the subject was working on the bicycle ergometer at a rate of 230 mkg/min. One series was made at the start of the work and lasted 3 minutes. The total oxygen requirement of the work was determined both in the normal condition and with the circulation to the legs blocked previous to work. In the latter case the circulation was restored immediately after cessation of work.

Table 1.

Normal start					Start with circul. to legs blocked				
Date	Excess O <sub>2</sub> during work liters	Excess O <sub>2</sub> in recovery liters	Total O <sub>2</sub> requirement liters	Net efficiency pCt.	Date	Excess O <sub>2</sub> during work liters	Excess O <sub>2</sub> in recovery liters	Total O <sub>2</sub> requirements liters	Net efficiency pCt.
16/4 ..	1.53	0.55	2.08	16.2	23/4 ..	0.84	1.75	2.59	13.0
18/4 ..	1.54	0.45	1.99	16.9	24/4 ..	0.85	1.42	2.27	14.8
25/4 ..	1.46	0.49	1.95	17.3	26/4 ..	0.87	1.73	2.60	12.9
20/4 ..	1.66	0.34	2.00	16.8	1/5 ..	1.03	1.64	2.67	12.6
2/5 ..	1.56	0.73	2.29	14.7	3/5 ..	0.94	1.46	2.40	14.0
4/5 ..	1.52	0.57	2.09	16.1	14/5 ..	0.96	1.32	2.28	14.8
mean	1.55	0.52	2.07	16.3	mean	0.92	1.55	2.47	13.7

The results of these experiments are tabulated and averaged in table 1. The table shows that the total oxygen requirement for performing  $3 \times 230$  mkg of work is considerably greater when part of the muscles have been working in an anaerobic condition than when they perform the work under ordinary conditions. The net efficiency of the work is 16.3 pCt in the normal condition but only 13.7 pCt when part of the work was done anaerobically. The deficit due to the blocking of the circulation must be  $1.55 - 0.92 = 0.63$  liters of oxygen, and the extra oxygen taken up after the restoration of the circulation is on an average  $1.55 - 0.52 = 1.03$  liters of O<sub>2</sub>.

In another series of experiments the blocking of the circulation to the legs was performed in the steady state of work. The work went on with blocked circulation at the same rate for 3 minutes, after which — during uninterrupted work — the circulation was restored. The work was now continued until the normal steady state value of oxygen-uptake was reached again, usually after about 10 minutes. The results are shown in table 2.

Table 2 shows that the oxygen uptake — less the resting consumption — in the steady state is on an average 0.56 l/min and that it decreases to 0.38 l/min when the legs are cut off from the circulation. 3 minutes of work in this condition give an oxygen deficit of 0.55 liters, but the oxygen uptake in excess of the steady state consumption during the subsequent recovery is 1.36 liters. The efficiency of the work if done first anaerobically, therefore,

is only  $\frac{0.55}{1.36}$  or on an average 43 pCt of the efficiency of work when this is done aerobically.

Table 2.

Date	Excess O <sub>2</sub> steady state liters/min	Excess O <sub>2</sub> blocked circul. liters/min	O <sub>2</sub> -deficit in 3 min. block. liters	Excess O <sub>2</sub> during recovery from block. liters	O <sub>2</sub> -deficit × 100 O <sub>2</sub> repaid	Net efficiency steady state pCt	Net efficiency of "anaerobic" work pCt.
15/5 .....	0.55	0.35	0.60	1.37	44	20.5	9.0
16/5 .....	0.59	0.39	0.60	1.57	38	19.1	7.3
17/5 .....	0.56	0.38	0.54	1.27	43	20.1	8.6
22/5 .....	0.51	0.37	0.42	1.85	23	22.1	5.1
23/5 .....	0.58	0.39	0.57	1.01	56	19.4	10.9
24/5 .....	0.58	0.39	0.57	1.08	53	19.4	10.5
mean	0.56	0.38	0.55	1.36	43	20.1	8.2

As the net efficiency of this work under aerobic conditions is 20.1 pCt, and assuming, the aerobic efficiency of the muscles that can be cut off from the circulation to be the same, one comes to the conclusion, that the efficiency of this work performed anaerobically with a delayed aerobic recovery is only 8 to 10 pCt.

In earlier experiments (ASMUSSEN, CHRISTENSEN and NIELSEN (1938), not published) the corresponding ratio, "anaerobic" efficiency: "aerobic" efficiency, was found to be: 0.57 in 5 minutes of work at 180 mkg/min (2 experiments), 0.60 in 2.5 minutes at 360 mkg/min (5 experiments) and 0.71 in 1.2 minutes work at 720 mkg/min (1 experiment).

Determinations of the blood lactates after restoration of the circulation showed an increase in 1 to 3 minutes to values of 35 to 40 mg pCt, then a gradual decrease to normal values after 10 to 15 minutes.

### c. The initial stages of work.

It is generally assumed, that the initial stages of work in which the oxygen intake does not cover the oxygen requirement, are performed partly anaerobically. Under such circumstances, and assuming the low efficiency of aerobic recovery after anaerobiosis found in the above experiments to be characteristic for anaerobic work, one might expect to find a lower efficiency or a higher oxygen requirement during the first minutes of work than in the steady state, where O<sub>2</sub>-requirement and O<sub>2</sub>-uptake are identical.

In order to test this assumption a series of experiments was performed in which the subject worked at rates ranging from

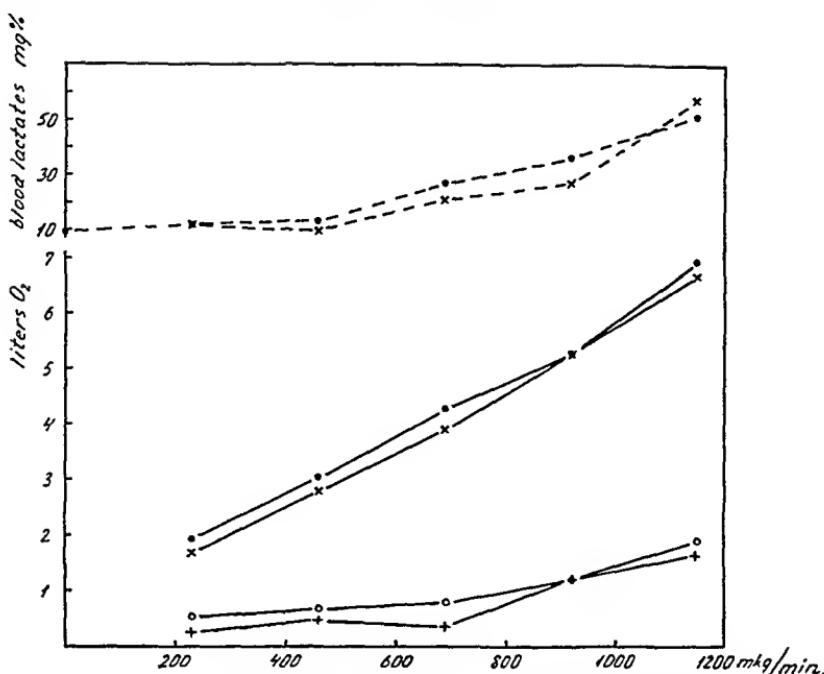


Fig. 2. Oxygen requirement for 3 minutes of work of varying intensity.

- Oxygen requirement computed from oxygen uptake of 3 min. at start, plus recovery.
- ×—× Oxygen requirement computed from oxygen uptake in the steady state.
- +—+ Oxygen deficit at start.
- Oxygen debt repaid in recovery
- Blood lactates after 3 min work
- ×---× Blood lactates in the steady state

230 mkg/min to 1150 mkg/min. The total oxygen uptake of work and recovery — less the resting consumption — of the 3 first minutes of work was compared with the excess oxygen uptake of 3 minutes of work in the steady state. 2 to 6 experiments at each grade of work were made. The results are averaged and presented in fig. 2, which shows that at all grades of work except that of 920 mkg/min, the oxygen requirement for 3 minutes of work is greater in the initial 3 minutes of work than in 3 minutes of steady state. The different result at 920 mkg/min, where they are equal, was verified by repeated experiments, and no plausible explanation for this deviation from the rule can be offered. In fig. 2 the oxygen deficit at the start of work and the oxygen debt repaid during recovery are also presented. They show as might be expected that the differences in oxygen requirements of the initial stage and the steady state is due to the fact that the oxygen

debt repaid in recovery is greater than the oxygen debt contracted at the start of work. The two upper curves show the blood lactates 1) at the end of the 3 minutes of work in the start, and 2) in the steady state of work, *i. e.* after about 15 minutes of work. Any direct correlation of the blood lactates to either the oxygen debt or the difference between oxygen debt repaid and oxygen deficit at start is not disclosed.

There are certain sources of error, that might tend to make the differences found in these experiments too great. We have carefully tried to avoid them and shall list them here in order to show, that the results presented really, as we believe, depict the actual facts.

1. Active movements of the subject in order to attain the working position will tend to increase the initial  $O_2$ -uptake, thus making the calculated  $O_2$ -deficit too small. 2. Corresponding movements after cessation of work will increase the amount of  $O_2$  taken up during recovery. The combined effects of 1 and 2 will make the total  $O_2$ -uptake too high. We consequently have avoided all active movements, that not directly belonged to the work. 3. In the steady state the muscles have been "warmed-up" and, therefore, according to experiments by ASMUSSEN and BAJE (1945), are able to perform maximal work more efficiently. This would make the  $O_2$ -uptake in the steady state lower. We tried to heat the muscles of thighs and buttocks by short wave diathermy previous to the start of the 3 minutes initial work, but found no effect whatever on the amount of oxygen required for this small work. 4. In the first seconds of work the fly-wheel of the ergometer must be accelerated up to the speed used in the work. This will add to the work performed in the initial stages and make the efficiency look too low. We have calculated the work of acceleration and found it so low (about 25 mkg) that it can be neglected.

It therefore seems justifiable to conclude from the results of the above experiments that the oxygen requirement of 3 minutes of work as a rule is greater when it is determined from the total oxygen uptake of work and recovery than when it is calculated from the oxygen uptake per minute in the steady state. The difference is caused by the fact, that the oxygen repaid in recovery exceeds the oxygen deficit contracted at the start of the work.

A further consequence of this will be that the efficiency of a short spell of work must be lower than the efficiency of a work

lasting longer. This could be demonstrated in experiments in which the work lasted from 0.5 min to 4 à 5 min. Two such series of experiments were performed, one with the work intensity of 690 mkg/min and one with 1,150 mkg/min. The results of these experiments are presented in figs. 3 and 4.

It will be seen that with the exception of the very short work of 0.5 min duration the different values of the total oxygen uptake of work and recovery lie on a straight line which, however, does not pass through the zero point. A mathematical expression for the two lines in our experiments will be: total  $O_2 = 1.30X + 0.15$  for the work of 690 mkg/min and: total  $O_2 = 2.17X + 0.25$  for the work of 1,150 mkg/min where X represents the duration of work.

The oxygen requirement per minute will consequently be:

$$O_2/\text{min} = 1.30 + \frac{0.15}{X} \text{ liters.}$$

$$\text{and: } O_2/\text{min} = 2.17 + \frac{0.25}{X} \text{ liters.}$$

The curves corresponding to these expressions are drawn in the upper part of figs. 3 and 4, and around them are seen the actually determined values of oxygen consumption per minute of work.

The curves as well as the formulas show, that as time (X) increases the oxygen requirement per minute work will diminish, approaching a value of 1.30 l/min and 2.17 l/min, respectively. These values, of course, are the steady state values, as was also confirmed by experiments. (Steady state values found: 1.30 l/min and 2.16 l/min, respectively.)

The equations are not valid when the time is very short, as the oxygen requirement of work lasting 0 minutes of course is 0. For the very small values of X the oxygen stores of the organism may play a rôle, making the work less anaerobic than later on. This is maybe the explanation of the relatively low values of total oxygen requirement for works of 0.5 min duration in figs. 3 and 4.

From the  $O_2/\text{min}$  the net efficiency can be calculated. With an assumed caloric value of oxygen of 4.8 it is found to vary in both series from about 22 pCt at the shortest work up to about 25 pCt for work of 5 min. duration.

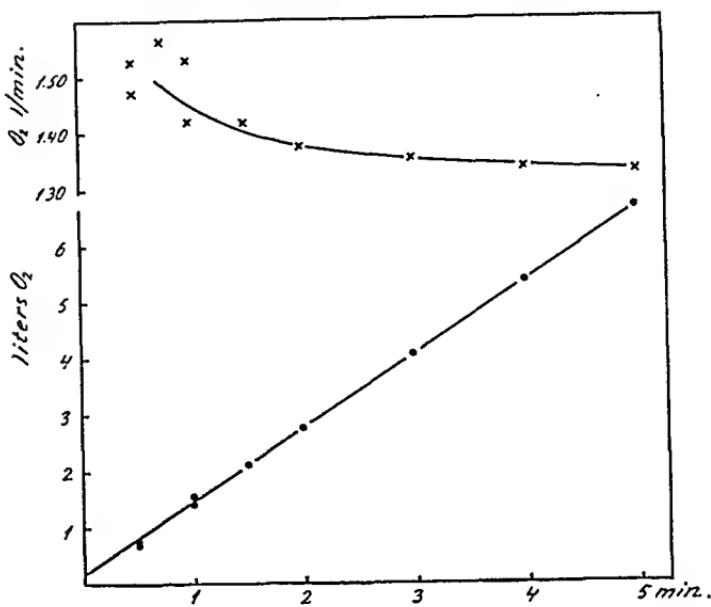


Fig. 3.

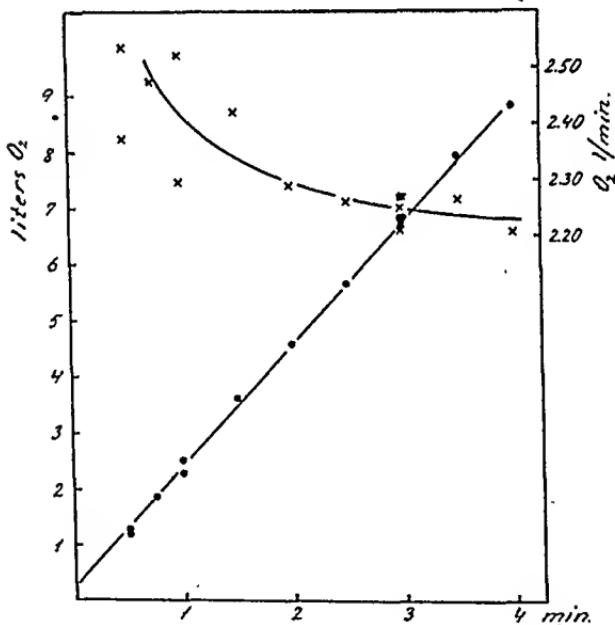


Fig. 4.

Figs. 3 and 4. Total oxygen requirement (●—●), and oxygen requirement pr. min work (×—×) for work of varying duration. Intensity of work: 690 and 1,150 mkg/min., respectively.

### Discussion.

The general conclusion that can be drawn from the experiments in which the circulation to the legs was blocked, is that when the energy production in rest or during work is forced to proceed via anaerobic processes, with a delayed aerobic recovery, the total costs in oxygen are greater than when the energy is liberated by simultaneous oxydative processes.

In the *rest* experiments the difference between oxygen deficit during blockade and excess oxygen uptake during recovery is first apparent when the circulation to the legs has been stopped for more than 5 to 6 minutes. The deficit up to this time will be about 100 cc of oxygen, and it seems justifiable to believe that it is covered by the oxygen stores of haemoglobin and myoglobin available in the legs. For periods of occlusion lasting more than 5 to 6 minutes true anaerobic conditions must prevail, and the energy necessary to maintain life must be derived from anaerobic processes. Of what kind these are cannot be decided, but the slight increase in the blood lactates after about 20 minutes of occlusion suggests that a glycogen breakdown has occurred.

When the blocking of the circulation to the legs was performed *during work* the energy requirement is considerably greater, and practically all of it must be derived from anaerobic break-downs, presumably also of glycogen as the increased blood lactates indicate. The oxydative recovery demands a comparatively large amount of energy as judged by the high excess oxygen intake during recovery. A production of mechanical energy that follows the line: anaerobic break-down → aerobic recovery, therefore, seems far less efficient than one in which energy is derived by a more direct oxidative process. The efficiency of the first process is — judging from these experiments — only 40 to 70 pCt of the efficiency of the latter.

This result may be compared with the results from myothermal measurements showing that the "initial heat" and the "recovery heat" are of the same order of magnitude, *i. e.* that the oxidative resynthesis of substances broken down anaerobically has an efficiency of only about 50 pCt (MEYERHOF (1930)). The efficiency of a muscle working anaerobically with a subsequent aerobic recovery is consequently only about half as great as the efficiency of anaerobic work. It therefore seems justifiable to conclude from

our experiments that the anaerobic processes — at least the "laetacide" ones — are shunted out from the recovery metabolism of muscles working aerobically under normal conditions, and that the oxidative energy is used more directly in rebuilding the potential energy, thus enabling the muscle to work with the same high efficiency as in the anaerobic condition. The discrepancy which existed between the absolute values found for the net efficiency of isolated frog's muscles and the efficiency determined in work experiments on man will hereby disappear: Under the most favourable conditions the mechanical efficiency of frog's muscles working anaerobically was found to be 23 to 20 pCt, making the efficiency of anaerobic work with subsequent aerobic recovery 12 to 10 pCt. This value is far below the value usually found in experiments on man, but is of the same order of magnitude as found in the present experiments, in which the muscles actually worked anaerobically with a subsequent aerobic recovery.

Assuming the lag in oxygen uptake in the *initial stages of work* to be the sign of a partly anaerobic period of energy production one must expect to find an extra amount of oxygen taken up during the time of recovery after work. A recovery from anaerobiosis is possible during aerobic work, as we found in the experiments in which the circulation to the legs had been blocked, but neither in our experiments nor in those known from the literature are there any signs of a "hump" on the oxygen curve in the first minutes of the steady state. All available evidence seems to indicate that the paying-back is postponed till after cessation of work. A small part of the oxygen taken up after work will undoubtedly be used in building up the oxygen stores of the organism, another small part will be used by the heart and the respiratory muscles in the transitory stage, but the rest no doubt is used in building up the chemical stores of energy broken down anaerobically at the onset of work. The amount of oxygen taken up after the end of work was in our experiments (with one exception) at all grades of work larger than the oxygen deficit at the beginning of work as might be expected from the point of view put forward above. Corresponding results have been recorded a. o. by HERXHEIMER (1935) NIELSEN and HANSEN ((1937) in a curve) and quite recently by ESKILDSEN (1945). E. HANSEN (1934), on the other hand, claims that the oxygen deficit at the start and the oxygen debt repaid during recovery are exactly equal. Against his conclusions may be objected that the grades of work he investigated were too

severe, so that a true steady state was not attained: Both oxygen deficit and oxygen repayment increased with the duration of work. Secondly, he determined the oxygen debts by planimetry of curves drawn through points from several experiments. This no doubt in itself may give rise to errors, as even the most careful placing of the curve in relation to the individual points may depend on an estimation, and further it must be borne in mind that the oxygen uptake from the lungs at any given moment does not correspond to the simultaneous oxygen consumption of the muscles. Some factors will tend to make it too large (*e. g.* the arterIALIZATION of dépôt blood) while others will make it too small (*e. g.* the inevitable lag between oxygen usage in the muscles and return of the venous blood to the lungs). The form of the curve representing the pulmonary oxygen uptake, upon which depends the areas measured by planimetry, thus becomes less well defined. We are inclined therefore, to believe that the small differences between oxygen deficit and oxygen repayment have been overlooked in the experiments of E. HANSEN, and the more so as the consequences of a larger oxygen repayment *i. e.* the lower efficiency of work of short duration as compared with longer lasting work. becomes evident from a scrutiny of E. HANSEN's data.

The suggestion that the efficiency of work is lower for short spells of work was first put forward by SIMONSON and HEBESTREIT (1930), later advocated by SIMONSON and SIRKINA (1934). The increase in efficiency found was, however, very considerable, 200 to 600 pCt, and serious objections against their choice of work have been raised by E. HANSEN (1933) as well as by CROWDEN (1934). Both E. HANSEN and CROWDEN could show that the net efficiency of a short spell of work is practically the same as for work of longer duration, but as mentioned above, a scrutiny of E. HANSEN's results discloses a difference in the efficiency of the same order of magnitude as found in the present experiments although this difference was not statistically significant. In CROWDEN's experiments — with one exception in which the trend was the same as in our experiments — the technique of work (intermittent work compared with continuous work) no doubt will make it utterly hard to demonstrate small differences as those found in the present experiments.

It seems justifiable, therefore, to conclude that the oxygen repaid during recovery after light and moderate work is slightly larger than the debt contracted at the start of work, thus making

the efficiency of short spells of work lower than the efficiency of work of longer duration.

It must be expected that in very short and severe work, in which the anaerobic part of the work is more dominant the efficiency will be considerably lower. An indication of this may be seen in the upward slope of the curve representing the oxygen requirement of running at increasing speeds presented by HILL (1926) although in such cases also the increased work of stabilization and of respiration etc. will play an important rôle.

### Summary.

The efficiency of the oxidative recovery after anaerobiosis has been studied. The anaerobic conditions studied were those prevailing in the legs when the circulation is cut off by means of pneumatic cuffs round the thighs, and the partly anaerobic conditions occurring in the initial stages of work.

It was found that both in rest and in work the oxygen repayment after the circulation to the occluded legs has been restored is considerably in excess of the oxygen debt contracted during the occlusion. The efficiency of work in which the energy is produced anaerobically with a subsequent aerobic recovery is therefore low; judging from these experiments only 40—70 pCt of the efficiency of aerobic work.

It is concluded from this that the anaerobic phases — at least the formation of lactic acid — in the after-contraction metabolism of muscle is shunted out during aerobic work, the oxydation energy being utilized more directly in recharging the contractile mechanism. In this way the loss in efficiency of about 50 pCt which a delayed aerobic recovery involves is avoided. There is no discrepancy between the values of efficiency found in experiments on frog's muscles and on human subjects.

In agreement with this it is found that the excess oxygen taken up after cessation of a work in which a steady state could be reached, is slightly greater than the oxygen deficit contracted at the start of the work. A consequence of this is that the efficiency as computed from the total oxygen uptake of work and recovery, is lower than the efficiency calculated from the steady state values, and further that the efficiency of a short spell of work is slightly lower than the efficiency of work of longer duration.

It must be expected that very severe work, which is performed mainly anaerobically with a subsequent aerobic recovery, has a low efficiency.

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## Interaction Between Fibrinogen and Polysaccharide Polysulfuric Acids.

By

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During investigations on the properties of synthetic polysaccharide polysulfuric acid esters (PIPER 1945 b), it was observed, that some of the substances precipitated the fibrinogen in plasma. As this was rather unexpected we have made an investigation on the interaction between proteins and polysaccharide polysulfuric acids. Some of the results obtained so far have been mentioned briefly by ASTRUP and PIPER (1945 b).

The substances investigated were prepared as described previously (ASTRUP, GALSMAR and VOLKERT 1944, ASTRUP and PIPER, 1945 a).

### Experimental.

#### A. Fibrinogen in Plasma and Cellulose Sulfuric Acid.

The first problem was whether the precipitation of fibrinogen by means of cellulose trisulfuric acid was quantitative. For analytical investigations of proteins this would be of great significance, as cellulose trisulfuric acid contains neither nitrogen nor phosphorous. A sample (C-12) of a cellulose trisulfuric acid insoluble in concentrated salt solutions was used. Citrate plasma was used (9 parts of blood + 1 part of 3.5 per cent sodium citrate).

Table I shows two important things. 1) No precipitate is obtained in citrate plasma, when adding a sufficient surplus of C-12. 2) The resulting solution forms no precipitate when heated to 56° for 10 min., from which follows that the fibrinogen present is influenced by a surplus of cellulose sulfuric acid in such a manner that it is not denatured by heating as it usually is. The absence of a precipitate after heating of the centrifuged solutions (after precipitation with C-12) is therefore no indication of a

Table 1. (Exp. 3).

To 2 ml citrate plasma are added 2 ml of solutions of C-12 (as sodium salt).

Resulting concentration of C-12 in per cent	After standing for 15 min.	After centrifuging and heating of the liquid to 56° in 10 min.
1	water clear	almost clear
0.5	turbid	slightly turbid
0.2	turbid and precipitate	, ,
0.1	bulky precipitate	, ,
0.05	, ,	, ,
0.01	precipitate	, ,
0.001	turbid	turbid and precipitate
0.0001	clear	, ,

quantitative removal of the fibrinogen with the precipitate, as previously assumed (PIPER 1945 b).

In Table 1 a concentration of about 0.1 per cent of C-12 gives the best conditions for the precipitation, but further experiments show that the amount of fibrinogen precipitated, measured in terms of the nitrogen content of the precipitate, varies from sample to sample even under equal conditions. The influence of the medium on the precipitation was therefore studied. It was thus found that dilution of the plasma with physiol. NaCl, or still more with water, decreases the amount of precipitate formed and eventually completely inhibits its formation. The same was the case when increasing the salt content of the solution, Table 2 and 3.

These experiments show that the precipitation is influenced to a large extent by the composition of the mixture, and that this may be the cause of the unsatisfactory results. Other substances were therefore tried and compared with C-12.

Table 2. (Exp. 16).

To 1.2 ml citrate plasma are added 1.2 ml of  $H_2O$  or solutions of NaCl and then 0.24 ml of 1 per cent C-12. Standing for 10 min.

Solution	Result
$H_2O$	bulky precipitate
0.5 per cent NaCl	, ,
1.0 , , ,	, , ,
2.0 , , ,	clear
4.0 , , ,	,
10.0 , , ,	,
20.0 , , ,	,

Table 3. (Exp. 18).

To 1 ml citrate plasma is added varying amounts of water and 0.5 per cent solution of C-12. Standing for 10 min. First section with a constant amount of C-12, second section with a constant concentration of C-12.

H <sub>2</sub> O	0.5 per cent C-12	Result
0 ml	0.2 ml	bulky precipitate
1 "	0.2 "	" "
2 "	0.2 "	" " , turbid
3 "	0.2 "	precipitate, turbid
4 "	0.2 "	" "
5 "	0.2 "	turbid
8 "	0.2 "	clear
0 ml	0.2 ml	bulky precipitate
1 "	0.4 "	" "
2 "	0.6 "	precipitate
3 "	0.8 "	clear
5 "	1.2 "	"
8 "	1.8 "	"

#### B. The Precipitation of Fibrinogen in Plasma by Means of Other Substances.

The other substances tried were Liquoid-Roche (Liq. R.), a starch polysulfuric acid (S-3), a cellulose sulfuric acid soluble in concentrated salt solutions (K-83.3) and a sulfuric acid ester of cellulose glycollic acid (K-81.1). Chitin sulfuric acid yielded no precipitate and was therefore not investigated. Rabbit citrate plasma was used throughout this investigation. The following symbols were used: 0 = clear, 1 = slightly turbid, 2 = turbid, but no precipitate, 3 = minor precipitate, 4 = precipitate and 5 = bulky precipitate.

Table 4 shows that the ability to precipitate fibrinogen in citrate plasma is influenced to a different degree by the various substances used. The concentration of the substance is far more critical for Liquoid-Roche than for any other of the substances investigated. Neither S-3, K-83, 2 nor K-81, 1 yield the same amount of precipitate as C-12.

Dilution of the solution (keeping the concentration of the precipitating substance constant) yields smaller amounts of precipitate (Table 5) and the same is the case, when the amount of the substance added is kept constant.

An increased salt concentration inhibits the formation of a precipitate, Table 6, but here Liquoid-Roche seems less affected. If however MgCl<sub>2</sub> is used instead of NaCl a precipitate is formed in all samples (also the diluted), but only when the MgCl<sub>2</sub>-solution is added after the addition of the substance investigated. With the substances or plasma alone MgCl<sub>2</sub> yields no precipitate.

Table 4. (Exp. 22).

To one ml citrate plasma is added an amount of the substance dissolved in water and the appearance is observed after standing for 10 min.

Solution ml	percentage	C-12	Liq.-R.	S <sub>3</sub>	K-83.2	K-81.1
1.0	2.0	0	0	1	0	3
1.0	1.0	1	0	2	1	3
0.1	1.0	5	5	2	3	3
0.1	0.1	3	0	1	1	1
0.1	0.01	2	0	0	0	0
0.1	0.001	0	0	0	0	0

Table 5. (Exp. 24).

Plasma ml	Solution ml, 1 per cent	H <sub>2</sub> O	C-12	Liq.-R.	S-3	K-83.2	K-81.1
1.0	0.1	0	5	5	2	4	4
1.0	0.2	1.0	5	2	2	3	4
1.0	0.3	2.0	2	0	1	2	2
1.0	0.5	4.0	0	0	0	1	0
1.0	0.9	8.0	0	0	0	0	0

By addition of NaOH to citrate plasma a precipitate with C-12 is obtained even at pH 9.2, but at pH 9.7 the solution is almost clear. It is very unexpected that a precipitate is obtained at such an alkaline reaction. By addition of HCl to the plasma the amount of precipitate after adding C-12 decreases and at pH 5.5 the mixture is only turbid. At still higher degrees of acidity (pH 4.2) a precipitate again appears, this time due to a precipitation of plasma proteins by means of acid, probably in connection with the amount of C-12 present (see later). In oxalate plasma the precipitate appears as finer particles than in citrate plasma.

The precipitate formed by adding C-12 to plasma is almost completely redissolved by addition of a surplus of the substance. An exception is K-81.1, where no resolution seems to occur. This is also the case when diluting a precipitated plasma mixture with water. But when adding a surplus of sodium chloride solution, it is the precipitate formed by addition of Liquoid-Roche which does not redissolve. An addition of heparin in not too large amounts to plasma before adding C-12 does not inhibit the precipitation.

Table 6. (Exp. 26).

To one ml plasma is added 0.5 ml of different concentrations of NaCl and 0.1 ml 1 per cent solution of the substance.

NaCl-solution Percentage	C-12	Liq.-R.	S-3	K-83.2	K-81.1
0.0	5	2	2	5	5
1.0	5	3	2	3	3
2.0	3	4	2	0	1
5.0	0	" 4	0	0	0
10.0	0	3	0	0	0

C. *The Stabilisation of Fibrinogen in Plasma by Means of Polysaccharide Sulfuric Acids.*

As pointed out in section A the presence of C-12 in citrate plasma inhibits the denaturation and precipitation by heating to 56° of the fibrinogen not precipitated previously by the addition of C-12 to the sample. This was unexpected because fibrinogen is the most labile of the plasma proteins and one of the proteins which is most easily denatured. This phenomenon was therefore investigated more closely.

First the action of heparin was tried, and it was found that increasing amounts of heparin decreased the amount of precipitate formed after heating the solution to 56° for 10 min. The effect is, however, inferior to the effect of C-12, as a mixture of 1 ml citrate plasma and 1 ml 5 per cent heparin (»Leo«) still becomes turbid after heating, while addition of 1 ml 2 per cent C-12 yields almost clear solutions. The centrifugate from plasma (2 ml) with C-12 (0.2 ml 1 per cent solution) added is clear after heating to 56° (10 min.), but a slight precipitate appears after addition of saturated ammonium sulfate to 0.3 saturation of such a centrifugate (before heating) and also after dilution with water and acidulation with acetic acid. Such processes are therefore not inhibited by C-12, but neither are they specific for fibrinogen, blood serum showing the same properties. Chitin disulfuric acid (K-51) acts in a manner similar to that of heparin. Two substances, with neither fibrinogen precipitating properties nor anticoagulant activity, viz. a cellulose glycollic acid (K-96) prepared as described previously (ASTRUP and PIPER 1945 a) and a chitosan glycollic acid (K-152.12) to be described in a subsequent paper (ASTRUP, BARSØE and PIPER) were tried. None of these substances showed any definite inhibitory effects on the heat denaturation.

D. *The Precipitation of Purified Fibrinogen Solutions.*

In order to exclude interference with the precipitation of fibrinogen from other plasma proteins, purified fibrinogen solutions prepared according to ASTRUP and DARLING (1942) from ox plasma were next investigated. The fibrinogen solution was used diluted with physiol. NaCl in the proportion 1 : 3.

To 10 ml fibrinogen solution is added 0.1 ml 1 per cent C-12. A bulky precipitate is formed and removed by centrifugation. Addition of ammonium sulfate (0.3 saturation) to the solution yields no precipitate, and the same is the case after heating to 56° for 10 min. Addition of NaCl (1 ml 10 per cent NaCl to 2 ml fibrinogen solution and 0.2 ml 1 per cent C-12) inhibits the formation of a precipitate, and a precipitate formed previously is redissolved. The addition of serum instead of physiol. NaCl inhibits to a certain degree the formation of a precipitate: 1 ml fibrinogen solution (diluted) + 3 ml physiol. NaCl + 0.4 ml 1 per cent C-12 → precipitate. 3 ml blood serum instead of 3 ml physiol. NaCl → only slight opalescence. But 3 ml plasma yields a precipitate; in this case, however, the amount of fibrinogen present is also increased.

A chitin disulfuric acid (K-51) precipitates fibrinogen from a fibrinogen solution, contrary to what is the case with fibrinogen in plasma (1 ml fibrinogen + 3 ml physiol. NaCl + 0.4 ml 1 per cent K-51). The same is the case to some extent with a chondroitin polysulfuric acid (K-74), which in fibrinogen solutions produces turbidity. Liquoid-Roche precipitates a fibrinogen solution. A surplus of cellulose trisulfuric acid (C-12) precipitates a fibrinogen solution, and the precipitate is not dissolved again when adding an excess of the substance (contrary to the conditions in plasma). Large amounts of heparin inhibit the formation of a precipitate (1 ml fibrinogen solution + 3 ml 0.75 per cent heparin → no precipitate or turbidity. 1 ml fibrinogen + 3 ml H<sub>2</sub>O + 0.4 ml 1 per cent C-12 → precipitate. 1 ml fibrinogen + 3 ml 0.75 per cent heparin + 0.4 ml per cent C-12 → no precipitate). The sulfuric acid ester of cellulose glycollic acid (ASTRUP and PIPER 1945 a) precipitates fibrinogen in plasma, but a similar substance prepared by KARRER, KOENIG and USTERI (1943), and kindly furnished us by Professor Dr. PAUL KARRER, Zürich, did not yield any precipitate in plasma, cf. PIPER (1945 b). In fibrinogen solutions, however, a precipitate appears. The cellulose glycollic acid itself produces no precipitate. At slightly alkaline reaction (phosphate buffer) no precipitation occurs when adding cellulose trisulfuric acid or chitin disulfuric acid, the pH value depending on the concentration of buffer salts present.

Thus purified fibrinogen solutions are more easily precipitated than fibrinogen in plasma. Heparin, which as mentioned inhibits the formation of a precipitate when cellulose sulfuric acids are added, also acts stabilizing on the heat denaturation of fibrinogen (1 ml conc. fibrinogen solution + 3 ml 0.75 per cent heparin → no precipitate after heating to 56° for 10 min.). Cellulose glycollic acid, which also yields no precipitate, but has no anticoagulant property, inhibits the denaturation only to a small extent. Fibrinogen itself in phosphate buffer at slightly alkaline reaction (pH ~ 8) is not precipitated by heating to 56° for 10 min.

#### E. Action on Other Proteins.

While an interaction between proteins (fibrinogen) and polysaccharide sulfuric acids at neutral or slightly alkaline reaction was unexpected, it was to be assumed that these high molecular acids would

react with proteins in general at slightly acid reaction in the same manner as nucleic acids and heparin. This proved also to be the case.

Ovalbumin (recrystallized) (1 ml) in 0.1-n acetate buffer (5 ml) begins to form precipitate at pH 4.4 when adding 1 ml 1 per cent cellulose trisulfuric acid (C-12) and at pH 4.1 when adding chitin disulfuric acid (K-51). A precipitated pig globulin under the same conditions begins to precipitate at pH 5.3 with C-12 and at pH 5.0 with K-51.

While the polysaccharide sulfuric acids protected fibrinogen against denaturation at 56°, rabbit serum (1 ml), with 3 ml 1 $\frac{1}{3}$  per cent heparin, C-12 or K-51 added, showed only insignificant differences when heated to 90° for 10 min. The inhibitory effect on the heat denaturation of other proteins is therefore not very pronounced.

### Discussion.

The experiments described indicate that the interaction between fibrinogen and polysaccharide sulfuric acids is of a rather specific nature. In general, high molecular acids only react with proteins on the acid side of the isoelectric points, this is the case of nucleic acids, E. HAMMARSTEN (1924), and heparin, FISCHER (1935), JAQUES (1943). While it is to be assumed, that other polysaccharide sulfuric acids than heparin act in a similar manner on proteins, it was unexpected that a reaction with fibrinogen also occurs on the alkaline side of the isoelectric point. This interaction was found to be highly dependant on the composition of the reacting medium, thus indicating a more loose combination between fibrinogen and the high molecular acids, than is the case in the compounds resulting from proteins and high molecular acids at an acid reaction, and which are salt-like products. The precipitate in question probably is a coacervate.

Now, fibrinogen is a protein of unusual properties originating from its structure as a thread-shaped protein of high molecular weight. HOLMBERG (1944) describes fibrinogen as a thread molecule with a molecular weight of about 700,000. The axial ratio of the molecule is 50. According to COHN and coworkers (1944) it has a molecular weight of about 500,000 with a length of 900 Ångström and a diameter of 33 Ångström (axial ratio = 30). The polysaccharide polysulfuric acids likewise are high molecular electrolytes. Presumably even at slightly alkaline reaction some positively charged groups on the fibrinogen molecule (arginine and lysine residues) may be able to combine with the negative charged polysaccharide sulfuric acid to such an extent that a precipitation

takes place, although the acidic groups (dicarboxylic acid residues) are negatively charged at this pH value. The presence of such charged groups in the compound formed may explain the dependence of the precipitation on the composition of the mixture as disclosed in the present investigations. The thread-shape of the molecules of fibrinogen and of the cellulose derivatives may facilitate the formation of the compound, while more globular proteins probably do not react to the same extent. Polysaccharide polysulfuric acids (or similar compounds of high molecular weight) of more globular configuration possibly may not precipitate fibrinogen in plasma. According to GRÖNWALL, INGELMAN and MOSIMANN (1945) heparin has a molecular weight of only 17,000, but shows a high »frictional ratio» (2,5) indicating an asymmetric shape.

In a previous paper, PIPER (1945 a), it was found that the synthetic polysaccharide polysulfuric acids agglutinate the blood platelets, and this phenomenon was later associated with the interaction between the polysaccharide derivatives and fibrinogen, PIPER (1945 b), but as it also was found that a substance (»Liquoid-Roche») was able to precipitate fibrinogen without agglutinating the blood platelets, this is not the whole explanation, even if the precipitation alone is sufficient to characterize such a substance as toxic. An excess of C-12 or K-51 still agglutinates the platelets, even if no precipitate is formed. A suspension of kaolin or bacteria is only agglutinated by C-12 in the presence of fibrinogen. Also the complement-inactivating properties of such substances may be taken into consideration, WILANDER (1939).

The fibrinogen precipitating properties explain why ASTRUP, GALSMAR and VOLKERT (1944) obtained straight parallel lines in investigations on the clotting of recalcified oxalated plasma containing various amounts of cellulose trisulfuric acid, while heparin, and to some extent also chitin disulfuric acid, yielded curved lines. Similar results were at the same time obtained by BERTRAND and QUIVY (1944) using »Liquoid-Roche». Curves of such appearance may be obtained when clotting plasmas containing various concentrations of fibrinogen (BARSØE and SELSØ).

While the fibrinogen precipitating properties of polysaccharide polysulfuric acids have not been known before, it was found by STUBER and LANG (1932) that »Liquoid-Roche» precipitates fibrinogen. The same was found by ZUNZ, MENA-UGALDE and VESSELOVSKY (1934, 1935), who observed a fall in the number of platelets. According to CHORINE (1941) fibrinogen is not the only protein of plasma, which is precipitated by means of Liquiod. Calcium ions are of importance for this reaction.

According to MYLON, WINTERNITZ and DE SÜTO-NAGY (1942) also protamin selectively precipitates fibrinogen from plasma, which is not quite consistent with our results concerning the action of elutropin sulfate on fibrinogen solutions (ASTRUP 1944). According to CHARGAFF and BENDICH (1943) ninhydrin and some naphthoquinone sulfonic acids produce a coagulation of fibrinogen resembling the formation of a natural fibrin clot.

FISCHER (1935) found that heparin, contrary to nucleic acids, protects proteins against denaturation by means of acids, and that even the heat denaturation may be hampered, although, according to COLDAHL and KAHLSON (1939), not to the same extent as by using other anticoagulants (chlorazol fast pink). Our investigations showed that the synthetic polysaccharide sulfuric acids only to a minor degree inhibited the heat denaturation of proteins.

These investigations were supported by the »P. Carl Petersens Fond», and the rabbits used were placed at our disposal by »Lovens kemiske Fabrik», Copenhagen.

### Summary.

The interaction between fibrinogen and polysaccharide polysulfuric acids is studied, and it is found that the formation of a precipitate is influenced to a large extent by the composition of the mixture (concentration, salt content, pH) and the nature of the substances used. Even on the basic side of the isoelectric point a precipitate is formed, which may be due to the properties of fibrinogen and the polysaccharide derivatives as thread-shaped high molecular substances. The precipitate formed probably is a coacervate. These observations explain the toxicity of the substances in question.

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## The Influence of g-Strophanthin on the Mechanical Properties of Cardiac Muscle.

By

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It is generally accepted that the glucosides of digitalis improve the activity of a failing heart. How this improvement is effected is still an unsettled question.

According to, among others, MACKENZIE (1918) and LEWIS (1937) the efficacy of digitalis is attributed to a depression of the auriculoventricular conduction. CHRISTIAN (1933), WENCKEBACH (1930) and others attribute the effect to an increase of the contractile power of the failing heart.

In the investigations of the latest years we find the same dualism. GOLD and CATTELL (1940) describe a clear positive inotropic effect of digitalis. KATZ, RODBARD, FRIEND and ROTTERSMAN (1938) and Mc MICHAEL and SHARPEY-SCHAFER (1944) do not find, however, any direct action of digitalis on the heart muscle. The last-mentioned authors attribute the beneficial therapeutic effect of digitalis to an action on the peripheral vessels, chiefly those of the liver. The divergent opinions derive from numerous experimental observations, mostly on hearts *in situ* or isolated hearts.

In experiments on such preparations there will come in several factors difficult to control, which will cause trouble in interpreting the results obtained. To be able to make a detailed analysis of the effect of digitalis on cardiac muscle, one must have a preparation as uncomplicated as possible. In these investigations we have used parallel threaded muscle bundles

(0.2—0.4 mm thick and 1.5—3 mm long) from the cardiac ventricle of a frog. Such a preparation in an oxygen saturated Ringer solution can be considered to work under perfect aerobic conditions. (CLARK, EGGLETON, EGGLETON, GADDIE and STEWART 1938.) *The purpose of these investigations is to analyse the effect of g-strophantin on the mechanical properties of "normal" cardiac muscle, that is strength of contraction, diastolic tension, elasticity, and viscosity.*

### Method.

The small muscle bundles are taken both from the apex and the base of the ventricle.

The muscle is prepared in ice-cooled Ringer solution under a binocular microscope (enlarging about 20 times).

The composition of the Ringer solution is as follows: 0.67 g NaCl, 0.02 g KCl, 0.04 g CaCl<sub>2</sub> + 6 H<sub>2</sub>O, and 0.02 g glucose in 100 cc aq. dest. To ensure a suitable colloidosmotic pressure 3 g dextran is added. (GRÖNWALL and INGELMANN 1944). The Ringer solution is buffered with NaHCO<sub>3</sub>, and then a stream of a mixture of 1 % CO<sub>2</sub> and 99 % O<sub>2</sub> is passed through, to a pH 7.2—7.4. The temperature of the solution 6°—7° is controlled thermo-electrically and remains constant throughout the experiment. The duration of contractions will thereby be sufficiently protracted to facilitate the determinations of the mechanical properties.

The muscle bundle is stimulated at constant intervals, 5 per minute, by means of a motor-driven switch in the outlet circuit of a multi-vibrator stimulation device which gives rectangular current pulses of different durations. In this case, as a rule, we have used impulses with a duration of 5 milliseconds. The silver tweezers holding the ends of the muscle bundle are used as stimulating electrodes and are electrically isolated from the recording apparatus.

A modification of the condensor-myograph devised by BUCHTHAL (1942) (fig. 1) has been used to record the variations of tension in the muscle. A movable condensor plate [(2) fig. 1] approaches a fixed condensor plate at increased muscle tension during contraction or at stretching. The capacity-variations are recorded by means of a high-frequency apparatus with amplifier and electrostatic oscillograph (fig. 2). The apparatus is also used for the determination of the dynamic elasticity in vibration experiments.

The investigations of the above mentioned properties are made at lengths varying between 100 % and 180 % of equilibrium length. The equilibrium length is the length at which the muscle, when stretched, just begins to develop tension. At higher elongations the muscle must consolidate for more than one hour after the stretch, before the examination of the mechanical properties can be performed. (LUNDIN 1944.) The

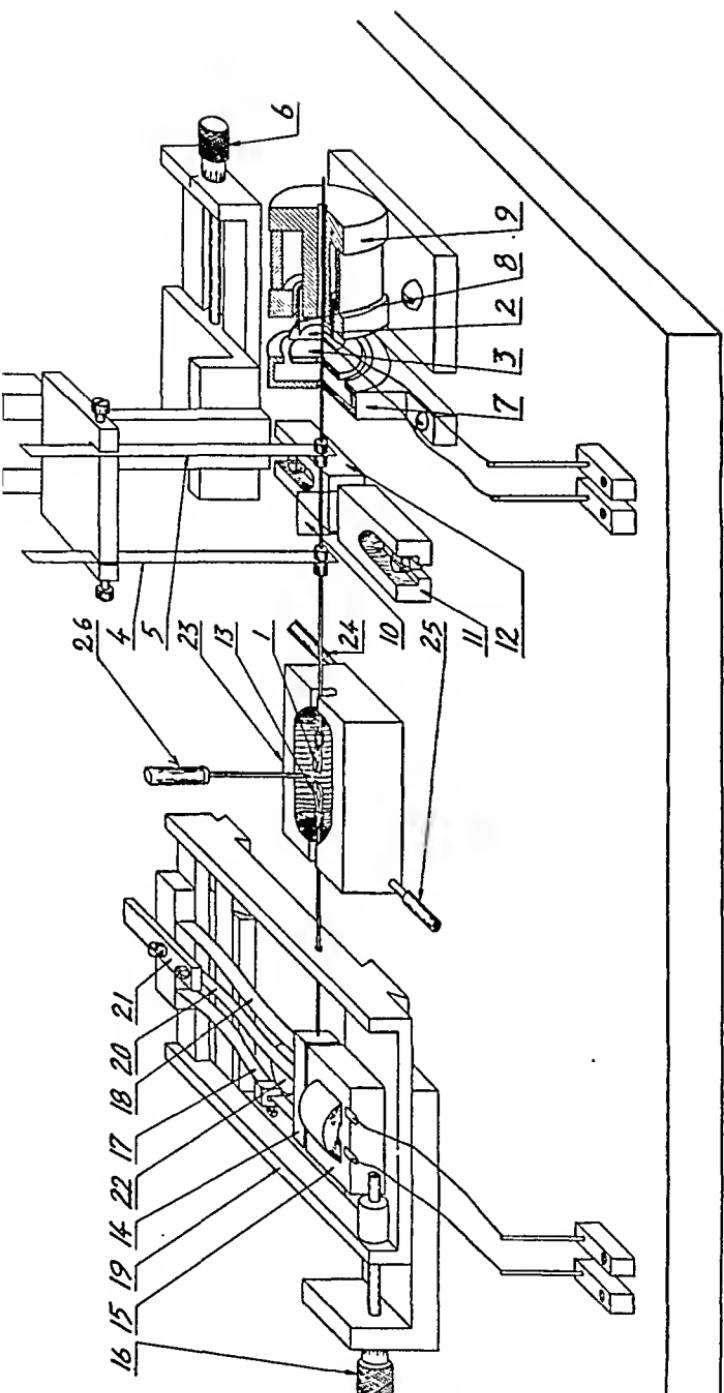


Fig. 1. Apparatus for registration of length-tension diagrams and measurements of dynamic stiffness.

(1) micro-tweezer. — (2) movable condenser plate in connection with (1). — (3) fixed condenser plate connected with grid of high frequency circuit. — (4) and (5) steel springs to keep tweezer (1) in position. — (6) micrometer screw by which distance between condenser plates (2) and (3) can be varied. — (7) screen for non-earthed condenser plate (2). — (8) coil attached to condensor plate (2). — (9) permanent magnet. — (10) thin mica disc gliding in oil between (11) and (12). — (11) and (12) brass blocks with adjustable distance for damping tweezer (1). — (13) micro-tweezer to hold other end of muscle fibre. — (14) iron bar holding micro-tweezer (13). — (15) electromagnet. — (16) micrometer screw to vary distance between (1) and (13). — (17) and (18) strong steel springs to hold iron bar (14) in front of electro-magnet (15). — (19) brass frame holding (14), (15), (16), (17) and (18). — (20) steel spring which by tightening moves iron bar (14). — (21) movable metal plate which can be clamped in different positions and thus spring to spring (20). — (22) oil bath to damp movements of tweezer (13). — (23) chamber with Ringer solution. — (24) and (25) tubes for cooling water. — (26) thermocouplement for temperature control.

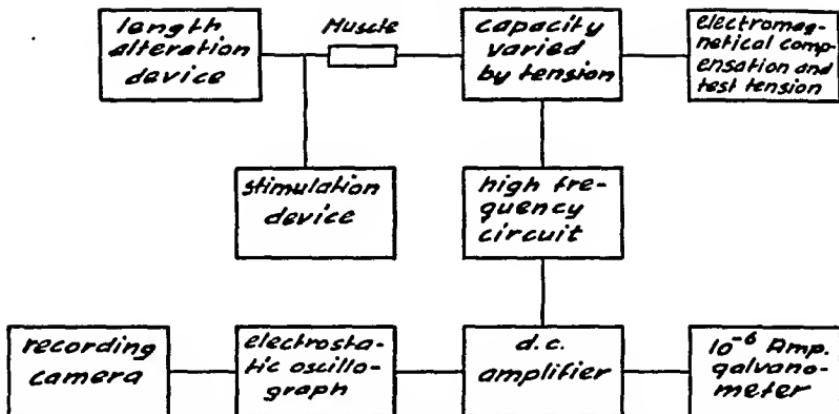


Fig. 2. Block diagram of apparatus for recording tension and stiffness.

deviation of the movable condenser plate caused by the stretching of the muscle is compensated by leading a current through the solenoid [(8) fig. 1] which moves in the field of a permanent magnet. The compensation current is read off and 4—5 contractions are recorded. The vibrations of the movable condenser plate are brought about by sudden current impulses in the solenoid (8), both at the height of contraction and at rest. The springs (4, 5) which carry the movable system, have such a stiffness, that during contraction the shortening of the muscle does not amount to more than 0.5 % of its total length. This length alteration is slight and the contraction can be regarded as isometric. Extra tension during the contraction is measured directly on the curves in millimeters, and an absolute measure is obtained by comparing the height of the contractions to the deviations from the baseline which are produced by current impulses corresponding to well-known loadings. The tension of the muscle at rest is given by the size of the compensation current. The total tension during the contraction is then the sum of the tension of the muscle at rest and the extra tension produced during the contraction.

In a number of experiments the muscle is slightly released from maximal tension during every other contraction. The decrease in length of the bundle which, in each experiment, is constant ca. 10—15 % of equilibrium length, is brought about by current variations in the electro-magnet [(15) fig. 1]. A camera, in which photographic paper is transported at constant speed, is used for the recording.

G-strophantin which has been chosen on account of its rapid effect, is used in concentrations varying between 1 part in  $5 \cdot 10^6$  and 1 part in  $2 \cdot 10^5$ . In the majority of the experiments the concentration 1 part in  $10^6$  is used.

At the concentration 1 part in  $5 \cdot 10^6$ —1 part in  $10^6$  several records are made, beginning 5 minutes after the addition of g-strophantin and finishing within 30 minutes. At higher concentrations records are taken for a period of 20 minutes.

## Results.

### Muscle tension at rest and during contraction.

*Tension at rest* is, with the exception of a few experiments with a great initial length, the same before as after the addition of g-strophantin. In a couple of experiments with an initial length of 180 % of equilibrium length a fall in tension after g-strophantin can be observed, but this fall is not greater than it can be explained as result of consolidation. In none of the experiments has a fall in tension appeared which can be interpreted as a diastolic effect of g-strophantin.

*Extra tension during contraction* does not increase after g-strophantin but remains unaltered. This is the case at all elongations investigated. The curve for isometric maxima coincides for drugged and normal muscles. This is also the case at lower g-strophantin concentrations at higher temperatures of the Ringer solution. As a criterion of the effect of g-strophantin we only have the toxic effect for the experiments in question. This effect has been observed in experiments with high g-strophantin concentrations 1 part in  $5 \cdot 10^5$  and 1 part in  $2 \cdot 10^5$ , where it has appeared after 15–20 minutes as a fall in the strength of contraction and an increase of the tension at rest. The changes in mechanical properties in this stage of the effect of g-strophantin are the objects of further investigations.

### *Stiffness at rest and during contraction.*

Stiffness ( $S$ ), which is the ratio between corresponding increase in tension and length  $\frac{\Delta \text{tension}}{\Delta \text{length}}$  and is measured in dyne  $\text{cm}^{-1}$ , is an expression of the elastic properties. In a body with such great variations in cross-section and length this quantity is more suitable than the elasticity modulus.

In our experiments the dynamic stiffness is determined by measuring the frequency of the recording system + muscle. The frequency of vibrations is adjusted so that it lies between 8 and 11 cycles per second.

When vibration time and mass of the system are known, the stiffness of the muscle ( $S_1$ ) can be calculated from the formula

$$S_1 = 4 \pi^2 \cdot \frac{m_0}{T_1^2} - S_0$$

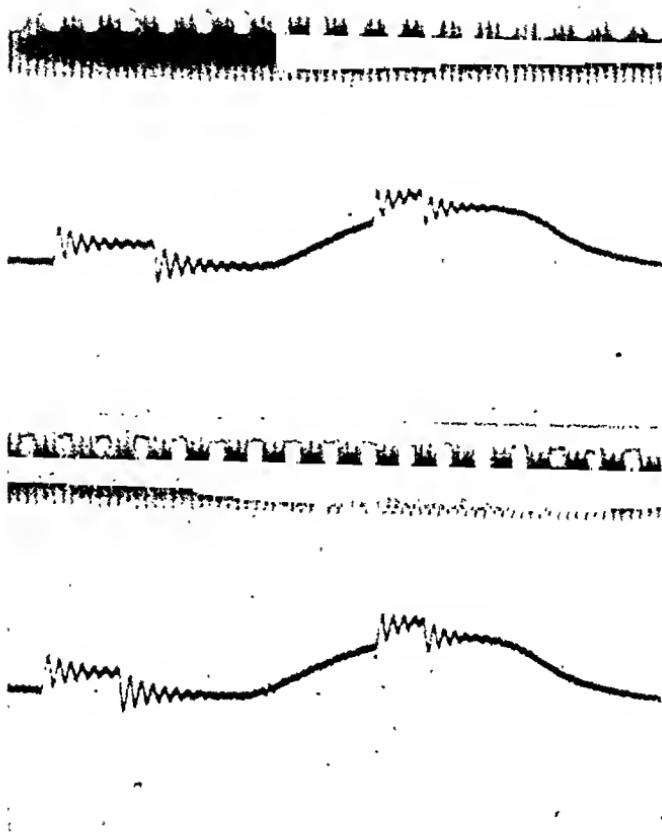


Fig. 3. Two contractions, the upper before g-strophantin, the lower 10 min. after addition of g-strophantin 1:10<sup>4</sup>. Time marks 1/50 sec.

where  $S_0$  = stiffness of the system without muscle,  $T_1$  vibration time for the system + muscle and  $m_0$  the mass of the vibrating system. The mass of the muscle can be ignored.

At least four contractions with vibrations are recorded each of them preceded by two vibration impulses at rest. The stiffness thus found at every registration is the mean of eight measurements. Stiffness is measured in 15 experiments both at rest and during contraction, with and without g-strophantin.

*Stiffness at rest* does not show any change after g-strophantin when referred to the same length, except in experiments at high initial lengths, where the consolidation is not yet finished. The fall in stiffness does not exceed that due to the fall in tension.

Hence stiffness during rest in proportion even to tension is not influenced by g-strophantin.

*Stiffness during contraction* is the same before and after addition of g-strophantin, both when referred to the same tension and the same length.

If we call the arithmetical mean of stiffness at rest before addition of g-strophantin 100, we get the following values for stiffness referred to the same length (15 experiments).

before g-strophantin		after g-strophantin	
rest	contraction	rest	contraction
100 ± 8 %	410 ± 3 %	97.7 ± 7 %	395 ± 3 %

As stiffness at rest and during contraction is the same when referred to the same tension and as g-strophantin does not alter the stiffness, we can conclude that also after g-strophantin stiffness is identical when referred to the same tension.

#### Viscous properties.

Decrement of amplitude in vibration in the stiffness experiments described above gives a measure of viscosity. If we call the proportion between two successive vibrations ( $f$ ), the so-called damping constant ( $P$ ) can be worked out from the formula

$$P = \log f^2 \cdot \frac{m}{T}$$

where  $T$  = vibration time in sec. and  $m$  = mass of the vibrating system in gram. This is an expression for total damping. To find the damping of the muscle ( $P_f$ ), the damping of the registering system itself ( $P_n$ ) must be subtracted from the total damping ( $P$ ).

$$\text{We get } P - P_n = P_f$$

Viscosity in cardiac muscle, expressed by the damping constant, when referred to the same length, is found to be the same before as after the addition of g-strophantin. As was the case in the stiffness and tension measurements in a couple of experiments at high elongations, we get a slight fall of viscosity at rest. This decreasing viscosity agrees with the picture we have got of the

changes in the mechanical properties by consolidation after an elongation.

The arithmetical mean of viscosity at rest before addition of g-strophantin being 100, we get the following values for viscosity referred to the same length, measured in 12 experiments.

before g-strophantin		after g-strophantin	
rest	contraction	rest	contraction
100 $\pm$ 12 %	382 $\pm$ 6 %	97 $\pm$ 12 %	362 $\pm$ 5 %

From the above experiments it is also obvious that viscosity referred to the same tension does not undergo any change after addition of g-strophantin.

If we release a muscle at rest or during contraction we get a deep fall in tension. The tension attained during release-contraction lies considerably below the isometric tension at the same length. This is due to the slow consolidation in cardiac muscle, and is an expression of its viscosity. The fall in tension of the muscle, referred to the same length and the same tension, is the same before and after the addition of g-strophantin. Thus the experiments with release-contractions show no changes in the viscous properties of the cardiac muscle produced by g-strophantin.

It appears from the above experiments, that on the preparation used, g-strophantin does not influence the diastolic tension or extra tension during isometric contraction, at a stage in the experiment when g-strophantin should have exerted its therapeutic action. Nor is stiffness at rest and during isometric contraction influenced by g-strophantin.

### Discussion.

It might be possible that other results could be expected in isotonic contractions due to changes in viscosity. A decrease in viscosity could increase the effect of the isotonic contraction, without any appreciable influence on the tension in isometric contraction.

However, viscosity, expressed by the damping constant, does not show any changes after addition of g-strophantin. This is further supported by the release experiments. The fall in tension during release gives an idea of the work in an isotonic contraction before and after g-strophantin. The decrease in tension is then

inversely proportional to the work developed. The fall in tension being the same before and after g-strophantin, we may conclude that g-strophantin does not influence the capacity of work.

Thus these investigations show that g-strophantin does not affect the mechanical properties of the normal cardiac muscle. Before we can draw any conclusions with regard to the therapeutic value of the drug, the experiments have to be completed with an investigation of hypodynamic cardiac muscle.

### Summary.

The mechanical properties of cardiac muscle have been examined on isolated parallel threaded muscle bundles under aerobic conditions, before and after addition of g-strophantin. G-strophantin does not influence any of the following properties: Diastolic tension, extra tension during contraction, stiffness at rest and during contraction, viscosity or the capacity of work during isotonic contraction.

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## Efferent Impulses in the Splanchnic Nerve.

By

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The paramount importance of the splanchnic area for the regulation of the arterial blood pressure was demonstrated by LUDWIG and his school (LUDWIG and THIRY, 1864, CYON and LUDWIG 1866) and has been repeatedly confirmed, *e. g.*, in modern times by JANSEN, TAMS and ACHELIS (1924) and KRAMER and WRIGHT (1932). CYON and LUDWIG also found that the effect on the blood pressure of electrical stimulation of the central end of the depressor nerve in the rabbit was greatly reduced after section of both splanchnic nerves or after compression of the aorta. In the dog, extirpation or severance of both ganglionic chains has been found to abolish or greatly reduce the pressor reactions after the clamping of the carotids (BACQ, BROUHA and HEYMANS 1934, SCHNEIDER 1934), and the analysis carried out by BERNTHAL, MOTLEY, SCHWIND and WEEKS (1945) has shown that the chemoreflexes as well as the pressoreflexes elicited from the sinus region have as their sole efferent pathway the thoracolumbar autonomies. In the cat, however, pressoreflexes may still be evoked, though to a reduced degree, from the sinus region, even after both the sympathetic ganglion chains have been extirpated (BACQ, BREMER, BROUHA and HEYMANS 1939). That the reflex action from the buffer nerves on the blood pressure is to a great extent exercised by altering the state of constriction of the vessels within the splanchnic area has also been observed directly. Thus electrical stimulation of the central end of the depressor nerve has been seen to cause a dilatation of the vessels

of the kidney and the intestine (for reference of HEYMANS, BOUCKAERT and REGNIERS 1933), and similar results have been obtained after eleetrical stimulation of the sinus nerve in respect of the vessels of the spleen, kidney, intestines and liver. Chemo-reflex reaetions from the earotid body have been found in the spleen (HEYMANS, BOUCKAERT, EULER and DAUTREBANDE 1932) and in the intestines (BERNTHAL and SCHWIND 1945). Corresponding effects have also been observed concerning the secretion of adrenaline (for literature ep EULER and LILJESTRAND 1934).

The buffer nerves are stimulated by the intraearotid and intraaortie pressure, as well as by the chemeial composition of the arterial blood, both of which, under physiologeal conditions, reflexly influence the circulation (cp EULER and LILJESTRAND 1942) and probably the adrenaline seeretion. There is a great difference, however, between the modes of action of these two kinds of stimuli. An increase in the intraearotid or intraaortie pressure leads to a rise in the number of impulses in the buffer nerves, as demonstrated by the action potentials, which in its turn is followed by a lowering of the heart rate, a vasodilation and a lessened seeretion from the adrenal medulla. Oxygen want or carbon dioxide aecumulation also elicit an increase in the number of impulses in the buffer nerves (cp EULER, LILJESTRAND and ZOTTERMAN 1939), usually of much smaller amplitude, but the ultimate results is quite the reverse: increased heart rate, rise of blood pressure and probably increased seeretion of adrenaline. The interpretation generally accepted is that the impulses evoked by stimulation of the pressor receptors cause an inhibition of the sympathetic centers responsible for vasoeonstriction, acceleration of the heart rate and adrenaline seeretion, whereas stimulation of the chemoreceptors calls forth increased aetivity of those centers. According to several authors (ep HEYMANS, BOUCKAERT and REGNIERS p. 39), stimulation of the central end of the depressor nerve not only causes a diminished tone of the vasoconstrictors but also an increase in the tone of the vasodilators. There will thus exist a kind of reciprocal innervation. A similar arrangement might be expeeted for the sinus mechanism, though no direct evidence seems to be available.

Since both presso- and chemoreceptors are constantly stimulated under physiologeal conditions, the resulting tone of the centers will be largely dependent on the relative magnitudes of their influences.

### Technique and Procedure.

All our experiments have been performed on cats in chloralose anesthesia, 0.06 g per kg body-weight being injected intravenously. The action potentials from the efferent fibres of the splanchnic nerve were recorded by means of an amplifier and the cathode-ray oscillograph previously described (ZOTTERMAN 1936).

*The Preparation.* After removing the skin just below and above the last rib, close to the vertebral column, the Muscul. latiss. dorsi and the oblique abdominal muscles were transected and drawn apart. By cutting through the inferior serratus posterior muscle parallel and close to the last rib, the splanchnic nerves were exposed where they pass over the diaphragmatic root. The nerve was laid free as far as its entrance into the coeliac ganglion, where it was transected. Care had to be taken at this moment that the nerve was not unduly stretched, as the cats exhibited very violent reaction to the nerve section. In order to reduce the signal-to-noise ratio, the common sheath of the greater splanchnic nerve was pulled off from the cut end. This procedure cannot, however, be applied to the lesser splanchnic nerve, as this nerve generally splits up into several thin fascicles some distance before entering the ganglion. In some preparations of the greater splanchnic nerve there was a very high spontaneous electric activity, obviously due to injury potentials set up by the afferent fibers. The behaviour of these fibres has been subjected to special study, which is reported separately (GERNANDT and ZOTTERMAN 1946).

The efferent fibers in the splanchnic nerve of the cat seem to be below 3 „ in diameter. Action potentials from single efferent fibers can thus be recorded only from very thin preparations which exhibit a high signal-to-noise ratio (cp ZOTTERMAN 1936). The electric response of the efferent fibers in our preparations consisted of summed up potentials, and thus the records did not permit of any direct counting of the impulse frequency. A further analysis could, however, be made by using an integrating device kindly placed at our disposal by Professor R. GRANIT. The amplified action potentials were then directly recorded by one ray of the oscillograph through one channel, while the other ray was driven by the integrator.

As the diaphragm was generally transected and the thorax thereby opened while preparing the nerve, artificial respiration had to be given by a Starling pump throughout the experiment. The arterial blood pressure was recorded from the femoral artery by means of a mercury manometer. Intravenous injections were made through the femoral vein. In some experiments the cats were bled from the femoral artery of the other leg. The blood was collected and heparinized for reinjection. In some other experiments the sinus nerves on both sides were exposed according to the method previously described (GERNANDT and ZOTTERMAN 1945).

## Results.

Efferent impulses were regularly found in the splanchnic nerve, though the intensity varied a great deal, which seems to be in good agreement with the well-known variations in the vascular tone. Sometimes the action potentials were continuous, but more often they occurred in groups, which might be synchronous with respirations or with the heart beats. Similar observations have already been described by ADRIAN, BRONK and PHILLIPS (1932) in different sympathetic nerves supplying constrictor impulses to blood vessels, and by BRONK, FERGUSON, MARGARIA and SOLANDT (1936) in the cardio-sympathetic fibres.

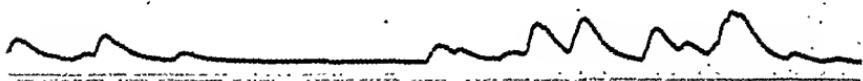
The simplest way of increasing the efferent impulses in the splanchnic nerve fibers is to stop the artificial respiration. The nerve activity now rises gradually in jerky stages, followed by a rise of the arterial blood pressure. After 45 to 60 seconds the activity reaches its maximum, and the records show that a synchronization of the fiber activity takes place (cp fig. 1 B). When artificial respiration is now resumed, the potentials quickly fade away, and after about 10 to 15 seconds there is a period of nearly complete silence for about 10 seconds, after which, in c. 30 seconds, the electric activity gradually returns to normal. This silent state was observed when the blood pressure had begun to fall but was still greatly elevated. A comparison with the noise level shows that the impulses were brought to a very low level.

During asphyxia, spontaneous respiratory movements became gradually stronger and sometimes so violent as to displace the electrodes. We therefore thought it worth while to establish a proper control, in order to ascertain whether the augmented and synchronized potentials observed during the course of the asphyxia were due to propagated efferent action potentials in the nerve and not either to movements of the electrodes on the nerve or to any secondary potentials in the nerve set up by muscular action potentials of the violently contracting diaphragm or other muscles over which the nerve passed. For that purpose we curarized three of the cats used in these experiments. The result was that the potentials appeared during asphyxia exactly as before curarization, although all muscular movements were completely inhibited.

A.



B.



C.



D.

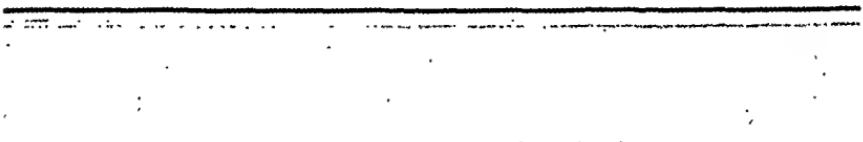


Fig. 1. Cat 4.3 kg, under chloralose anaesthesia. Action potentials from the splanchnic nerve recorded directly (upper curve) and by means of an integrator (lower curve). A. Standard ventilation air; B. Asphyxia for 1 minute; C. Standard ventilation air again for 10 seconds. D. Control. Nerve killed between the electrodes.  
Time 50 cycles per second.

If artificial respiration was established with a gas mixture poor in oxygen, the action potentials were greatly increased in magnitude (fig. 3), as was also the case when the air was replaced by 10 per cent carbon dioxide in oxygen (fig. 4 B). Thus both oxygen want and carbon dioxide accumulation increase the potentials. On the other hand, if oxygen was given instead of air, a small but definite decrease in the activity was observed. This must signify that, even with air, a certain stimulation from oxygen want occurs, which enhances the impulses in the splanchnic nerve.

Asphyxia could also be obtained by bleeding the animal, and the result was a very considerable increase in the impulses, though in this case the blood pressure was going down. It is striking that the effect was observed already 10—20 seconds after the bleeding had started. By retransfusion the picture could be transformed to its original state (fig. 5). A similar result was obtained when the blood pressure was lowered by the injection of acetylcholine (fig. 6 and 7), the change also appearing rather quickly in this too. On the other hand, if the blood pressure was raised by adrenaline (fig. 8), the impulses decreased and might nearly disappear. These observations seem to indicate that the effect of asphyxia is a combination of the stimulating action of the oxygen want and the carbon dioxide accumulation, on the one hand, and the inhibitory action of the elevated blood pressure, on the other.

In order to get a better insight into the mechanism responsible for the effects observed, the influence from the carotid sinus and the depressor nerves was studied. Clamping of the carotids gave an obvious increase in the potentials (fig. 9). As is well

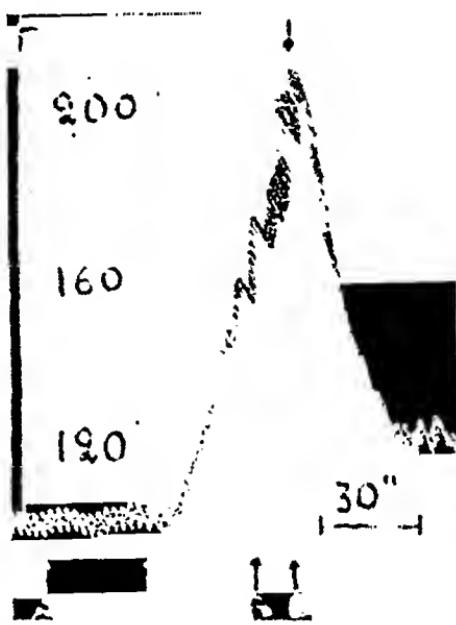
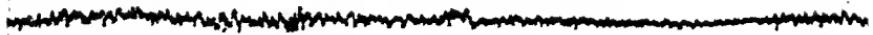


Fig. 2. Record of blood pressure during asphyxia from ↑ to ↓. Arrows A, B and C refer to the moments when the records A, B and C of fig. 1 were taken.

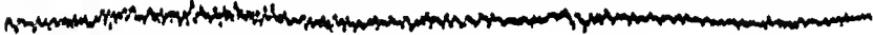
A.



B.



C.



D.

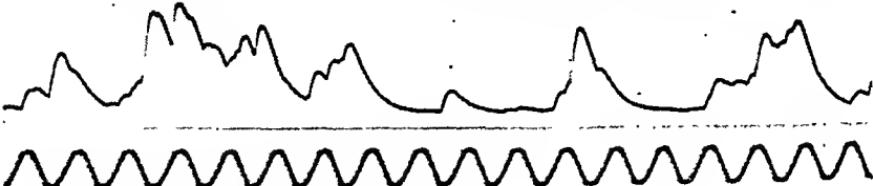
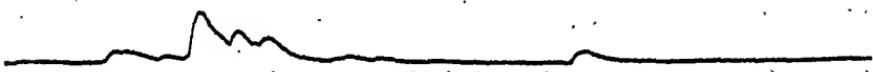


Fig. 3. The same cat as in previous figures. A. Standard ventilation air; B. Asphyxia for 1 minute. C. Standard ventilation air; D. Standard ventilation with 7.3 % O<sub>2</sub> in N<sub>2</sub>.

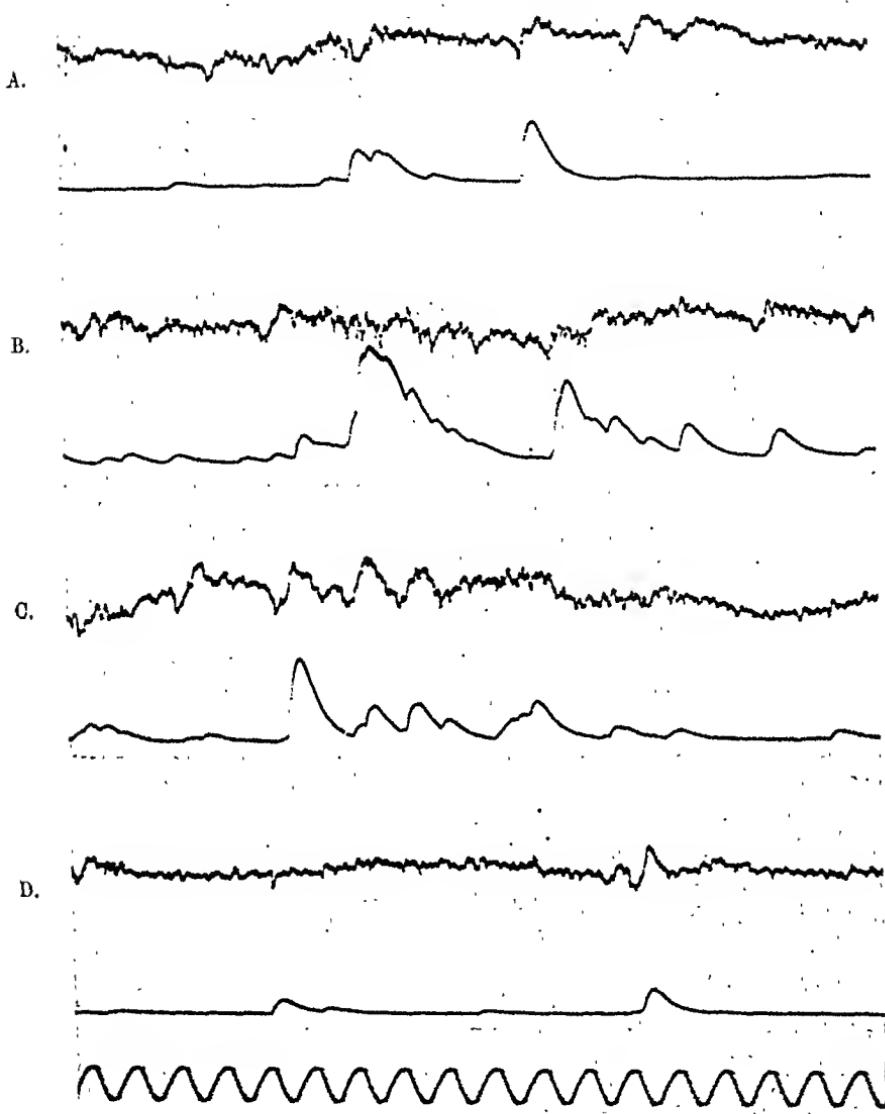
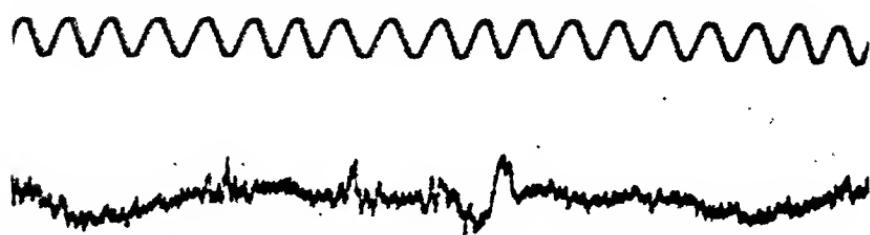


Fig. 4. Cat, 2.8 kg. A. Standard ventilation air; B. Ditto with 10.5 % CO<sub>2</sub> in O<sub>2</sub>; C. Standard ventilation air; D. Ditto with 100 % O<sub>2</sub>.

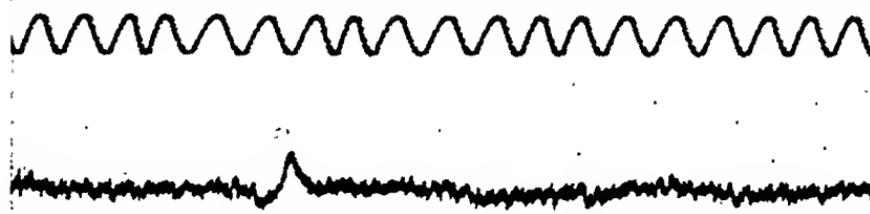
A.



B.



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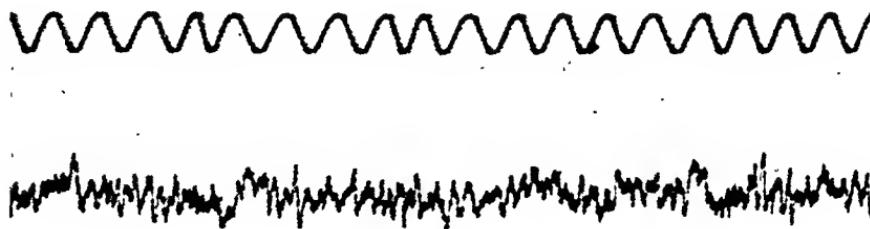


Fig. 5. Cat, 3.0 kg. A. Standard ventilation, BP 120 mm Hg; B. Bleeding from femoral artery, BP 40 mm Hg; C. The lost blood was heparinized and injected through the femoral vein, BP 140 mm Hg; D. Repeated bleeding of c. 25 ml blood, BP 40 mm Hg.

known from earlier experiments, this leads to a moderate rise in the number of potentials in the sinus nerve that are elicited by chemical stimulation (cp EULER, LILJESTRAND and ZOTTERMAN 1939), but a reduction of the potentials from the pressoreceptors.

B.

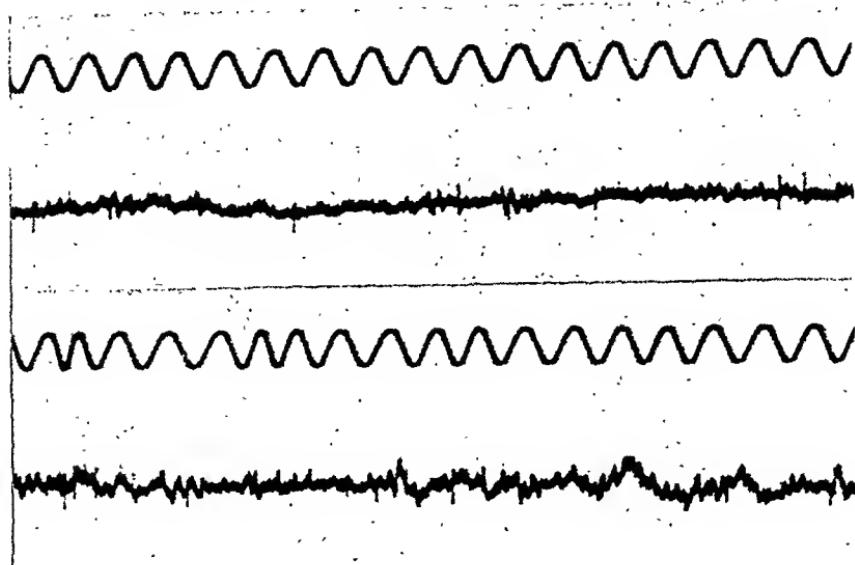


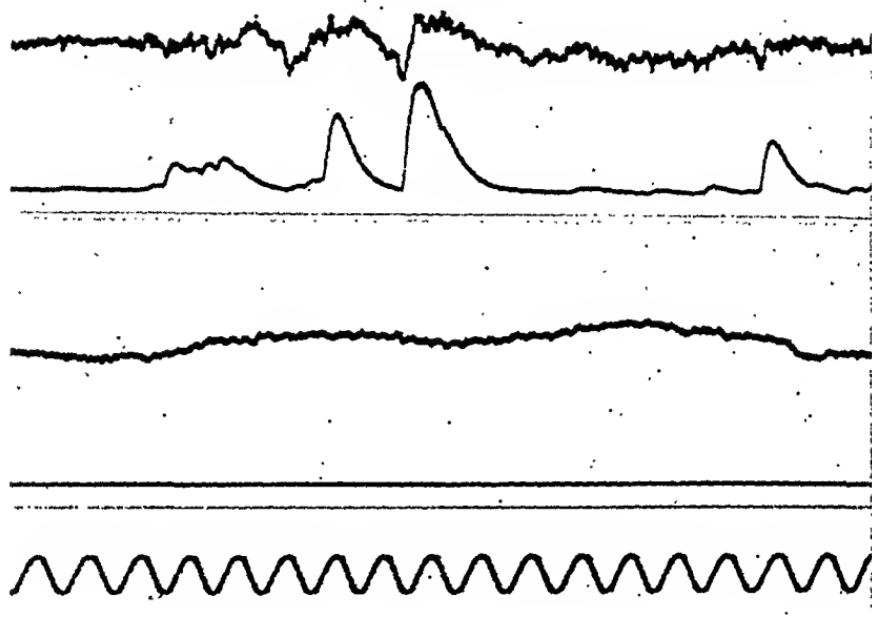
Fig. 6. Cat, 2.7 kg. A. Standard ventilation air BP 80 mm Hg; B. After injection of  $1.5 \gamma$  acetyl choline, BP 50 mm Hg.

Both these effects contribute to raising the blood pressure (cp EULER and LILJESTRAND 1943). The result obtained conforms well with these findings, and demonstrates the influence of presso- and chemoreflexes from the sinus mechanism on the activity of the splanchnic nerve under fairly physiological conditions.

Another proof of such an influence can be obtained, if the action potentials of the splanchnic nerve are recorded before and immediately after section of the sinus nerves and the vagi. In order to be able to compare the results, it is of course necessary to prepare the nerves beforehand and cut them without altering the position of the electrodes. As is illustrated in fig. 10, the effect of the operation was a great increase in the amplitude,

Fig. 7. Record of blood pressure when  $1.5 \gamma$  acetyl choline was injected intravenously  $\frac{1}{2}$ . Arrows A and B refer to records A and B in fig. 6.

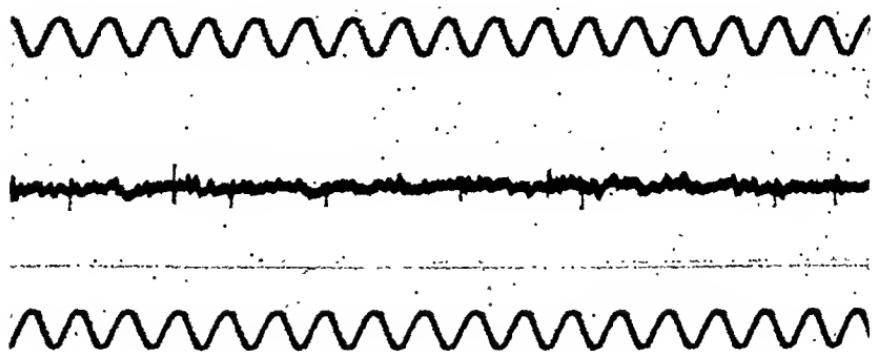
A.



B.

Fig. 8. Cat, 2.8 kg. A. Standard ventilation air, BP 150 mm Hg. B. After injection of  $10 \gamma$  adrenaline, BP 200 mm Hg.

A.



B.

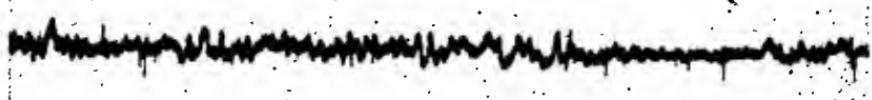


Fig. 9. Cat, 2.7 kg. A. Standard ventilation air, BP 90 mm Hg. B. Carotid arteries clamped, BP 100 mm Hg.

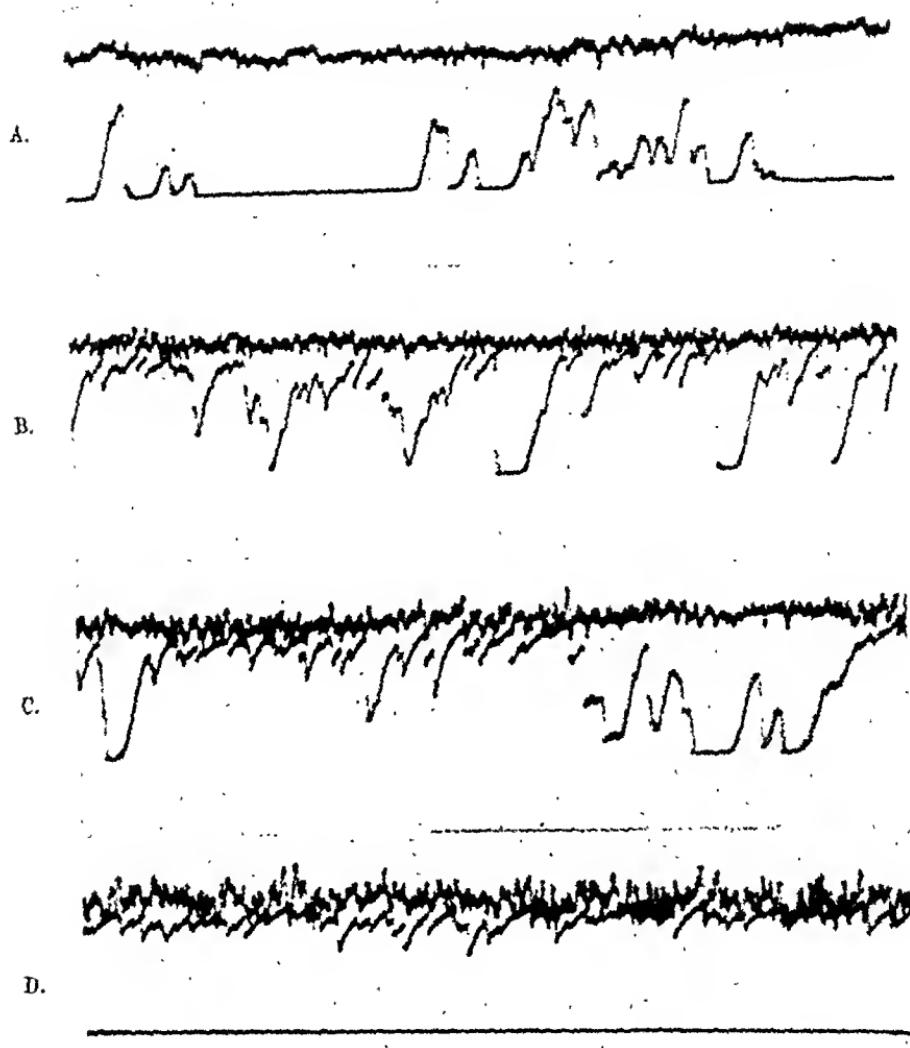


Fig. 10. Cat, 2.9 kg. A. Standard ventilation, BP 105 mm Hg; B. Asphyxia for 65 seconds, BP 200 mm Hg; C. Standard ventilation after the severance of all buffer nerves, BP 105 mm Hg; D. During asphyxia for 25 seconds, BP 115 mm Hg.

though the blood pressure in the experiment in question remained constant. There was still a further increase during asphyxia, as shown in fig. 10 D. But neither pure oxygen nor oxygen want had now any influence on the magnitude of the potentials, though a small increase was observed when 10 per cent carbon dioxide

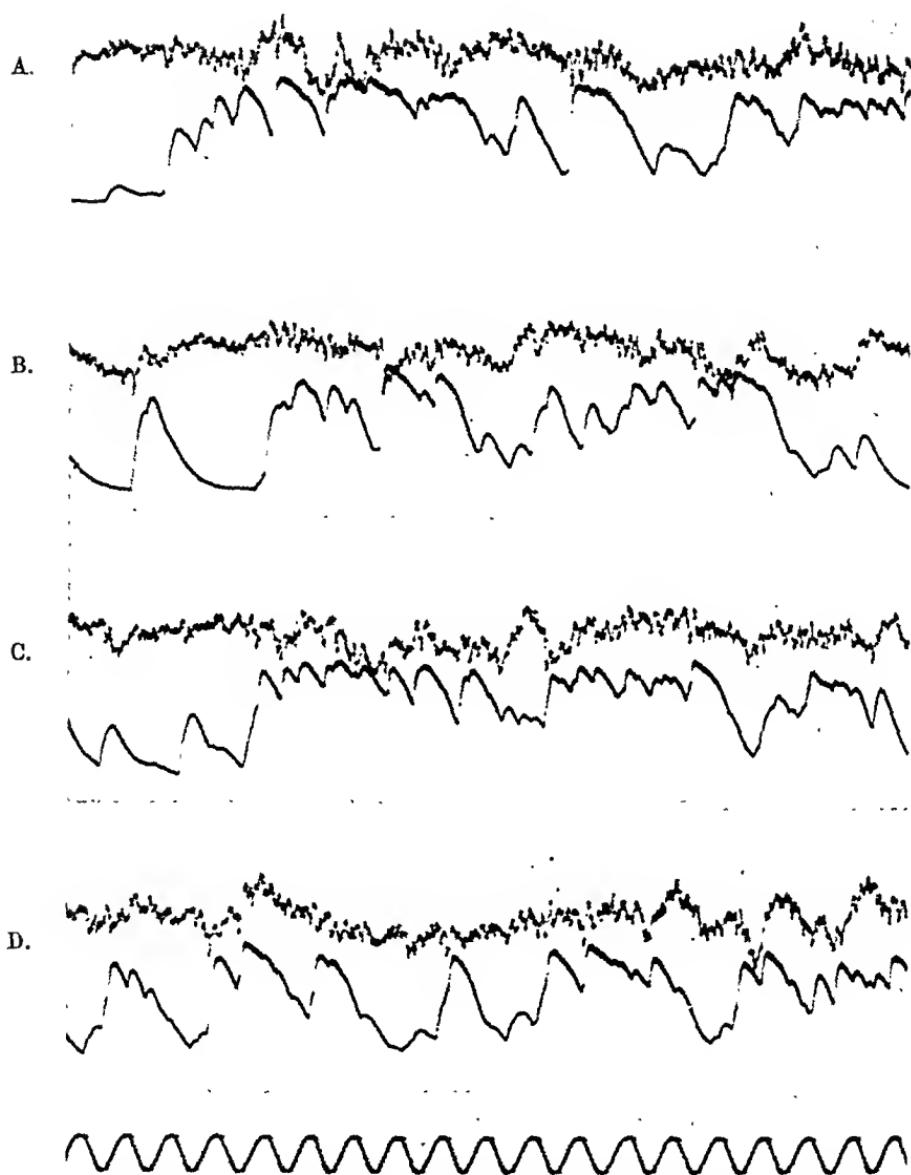


Fig. 11. Cat, 2.8 kg. After the severance of Hering's nerve and the vagus on both sides. A. Standard ventilation air, BP 240 mm Hg; B. Ditto with 7.3 % O<sub>2</sub> in N<sub>2</sub>, BP 130 mm Hg; C. Ditto with 10.5 % CO<sub>2</sub> in O<sub>2</sub>, BP 150 mm Hg; D. Ditto with 100 % O<sub>2</sub>, BP 200 mm Hg.

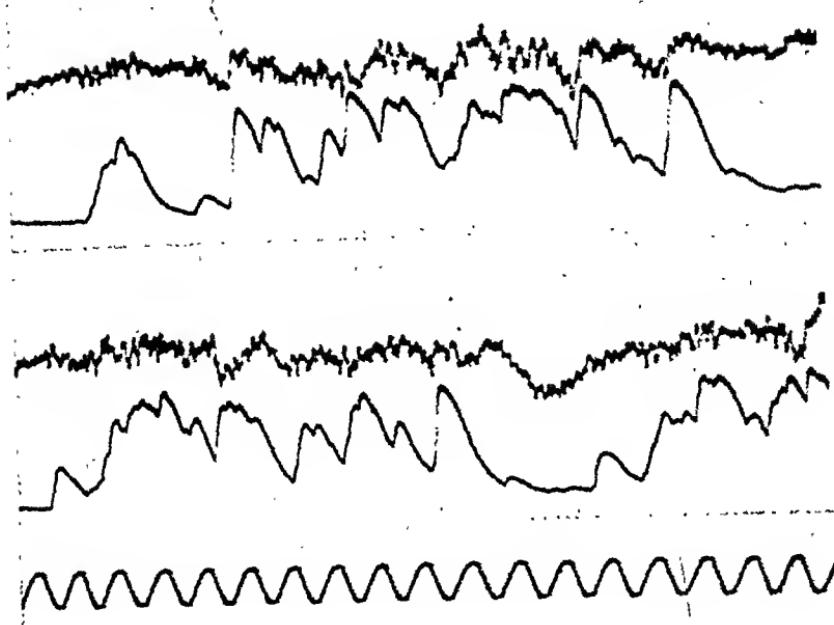


Fig. 12. Cat, 2.8 kg. Buffer nerves cut. A. Standard ventilation air, BP 140 mm Hg. B. Ditto after 10  $\gamma$  adrenaline intravenously, BP 240 mm Hg.

in oxygen was given (fig. 11). The effect of asphyxia after denervation thus seems to be due entirely to the accumulation of carbon dioxide. If the blood pressure was raised by adrenaline from 140 to 240 mm, no effect on the potentials was observed (fig. 12). In another case, however, a considerable decrease in the amplitudes was found after denervation, when the blood pressure after adrenaline rose from 50 to 100 mm.

#### Discussion.

The results obtained clearly show that the impulses in the splanchnic nerves are greatly modified by influences over the sinus and aortic mechanisms. These effects are exercised by alterations in the chemical composition of the blood as well as by variations in the blood pressure. In our experiments oxygen want was found to cause a very considerable increase of the impulses occurring during air-breathing, whereas a diminution was observed when oxygen was substituted for air. These effects disappeared, however, when the buffer nerves had been eliminated. The sti-

mulating action of oxygen want on the vascular center is therefore mostly indirect. This corresponds well with the effects of alterations in the oxygen pressure on the activity of the sinus nerve (EULER, LILJESTRAND and ZOTTERMAN 1939) and also on respiration, blood pressure and heart rate (cp EULER and LILJESTRAND 1942), though the results on circulation may be more or less obscured by the regulation via pressoreceptors. On the other hand, in our experiments, carbon dioxide stimulated the action potentials in the splanchnic nerve even after denervation, though probably to a smaller degree than before. This would imply that the action is exercised both reflexly and directly on the center. This seems to be in harmony with known facts about the influence of carbon dioxide accumulation on the sinus nerve activity and on respiration and blood pressure. It must be pointed out, however, that ALEXANDER (1945) observed an increase in the action potentials in the inferior cardiac nerve during oxygen want, even after isolation of the upper thoracic cord from all other nervous influences by low- and mid-thoracic transections of the spinal cord and section of all dorsal roots and of the sympathetic chains between those transections. The author himself points out that the possibility cannot be excluded that the activity in the inferior cardiac nerve in the deafferentiated spinal preparation might be due to irritative effects from the trauma of the cord.

With regard to the results observed by us during asphyxia, it is obvious that the stopping of the artificial respiration will lead to oxygen want and carbon dioxide accumulation, both of which will act on the sinus and aorta mechanisms, evoking impulses that stimulate the vasoconstrictor center. Carbon dioxide will also act on the center directly. As a consequence of these influences, the blood pressure rises, and now extra inhibitory impulses are elicited by the stimulation of the pressoreceptors, and these impulses work in a direction opposite to that of the chemical stimulation. The net result will be determined by the difference in effect between the two sets of impulses. When artificial respiration is reestablished, the chemical stimulation quickly disappears, but the blood pressure may still be elevated — a similar prolonged elevation of blood pressure due to inertia of the effector system has been described by PITTS, LARRABEE and BRONK (1941) — and inhibitory impulses will then remain strong. This seems partly to explain the fact that there is a short period just after the blood pressure is beginning to fall when the impulses

are less frequent than before asphyxia or even disappear entirely. Since PITTS, LARRABEE and BRONK have been able to demonstrate that increased discharge in the inferior cardiac nerve during hypothalamic stimulation is followed by an inhibition even after section of the buffer nerves, it seems very probable that a reduced excitability of the sympathetic center follows intense activity. The above-mentioned after-effect when artificial respiration is started again might therefore be caused to some extent by the result of this lowering of the excitability.

According to the experience of the school of HEYMANS (cp HEYMANS, BOUCKAERT and REGNIERS, p. 110), the vasomotor centers and the centers for the adrenaline secretion are themselves insensitive to physiological variations in the arterial pressure, whereas extreme reduction of the pressure may exercise a directly stimulating influence. This last effect must certainly be ascribed to chemical stimulation. When the blood pressure had been raised considerably from the normal level by adrenaline, we found that the activity of the splanchnic nerve disappeared more or less. This did not happen when the buffer nerves had been cut. The conclusion must be that the great reduction of the potentials in the splanchnic nerve is the result of a very strong inhibition from the pressoreceptors. But this is not the only effect. A certain reduction of the normal chemical stimulation is also to be expected. This would be in harmony with the corresponding immediate effect on respiration of adrenaline, involving a reduction that may even lead to apnoea. Most of this effect on respiration is due to the abolition of the chemical stimulation from the sinus and aortic bodies (cp GERNANDT, LILJESTRAND and ZOTTERMAN 1945), but a small part of the effect can be obtained after section of the buffer nerves and may be attributed to an improved circulation through the center, which will diminish the accumulation of carbon dioxide. A similar influence of adrenaline on the vasomotor center seems probable. This will especially be the case if the circulation is inadequate. Thus, when the blood pressure was very low, adrenaline was found greatly to inhibit the impulses after denervation of the sinuses and section of the vago-depressor nerves. Some small effect might also be expected when the circulation is adequate, and we note in this connection that after the elimination of the buffer nerves slight inhibiting effects of adrenaline on the action potentials of different sympathetic nerves have been observed by ADRIAN,

BRONK and PHILLIPS (1932), PITTS, LARRABEE and BRONK (1941), and by ALEXANDER (1945).

The comparatively great effect of bleeding on the action potentials of the splanchnic nerve is explained by the assumption that in this case asphyxia is accompanied by a lowering of the blood pressure, which in itself will reduce the inhibitory influence from the pressoreceptors. The same holds true for the effect of acetylcholine. Probably in both cases local asphyxia sets in rather quickly, which would explain the rapid development.

The assumption that the buffer nerves influence vasodilators as well as vasoconstrictors, would imply that, corresponding to a diminution of the efferent pressor impulses in the splanchnic nerve, and increase in the dilatory impulses appears. Thus, *e. g.*, at the height of adrenaline action or shortly after asphyxia, one might expect such impulses. We have been unable, however, to observe anything that can be attributed to such an activity. Of course there is the possibility that the fibers concerned might be much smaller than those leading to vasoconstriction. They might thus escape observations.

### Summary.

Efferent impulses were observed in the splanchnic nerve of the cat, sometimes continuous, sometimes synchronous with respiration or with the heart beats.

During asphyxia the amplitude of the impulses increased greatly. If artificial respiration was resumed, the electric activity quickly diminished and for a while nearly disappeared. Oxygen want led to an increase in the potentials, and pure oxygen to a decrease. Both these effects disappeared after the elimination of the buffer nerves. Accumulation of carbon dioxide caused greater activity in the nerve before as well as after section of the sinus and vagodepressor nerves.

Bleeding, or the injection of acetylcholine, led to a remarkable increase in the potentials, whereas adrenaline gave rise to a diminution or even the disappearance of the impulses. After denervation of the sinus and aorta mechanisms, the effect of adrenaline was very small at normal blood pressure but fairly great at low level.

Section of the sinus and vagodepressor nerves greatly increased the electric activity of the splanchnic nerve.

The results are interpreted as being due to reflex influence from the chemo- and pressoreceptors of the sinus- and aorta mechanisms and to a central effect from carbon dioxide accumulation.

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# The Effect of Respiratory Changes upon the Spontaneous Injury Discharge of Afferent Mammalian and Human Nerve Fibres.

By

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This paper deals with a spontaneous activity of nerve fibres which sometimes occurs in splanchnic nerve preparations from the cat. At first this phenomenon was a great hindrance only to the fulfilment of our original plan of work. It soon became clear, however, that the impulses were being discharged in afferent fibres. The further study of this spontaneous activity gave an explanation of the well known phenomenon of post-ischemic sensory tingling in the human subject after renewal of the blood flow to a previously compressed limb. It was also shown that carbon dioxide produced a great reduction of this tingling thus confirming an old suggestion made by one of us (ZOTTERMAN 1933), that the post-ischemic tingling was related to a lowering of the concentration of metabolites.

## Spontaneous Impulses in Afferent Fibres of the Splanchnic Nerve of the Cat.

In the course of our research on the efferent impulse-traffic in the splanchnic nerve of the cat (GERNANDT, LILJESTRAND and ZOTTERMAN 1946) we sometimes observed a more or less pronounced activity of the nerve consisting of fairly large spikes. These spikes occurred in a very strong continuous flow, in contrast to the more jerky outflow of irregularly shaped action-potentials of low amplitude which is characteristic of the efferent discharge of this nerve. The general shape of the fairly large

spikes indicated that they must derive from fibres of a diameter above  $4 \mu$ . A microscopic examination of the splanchnic nerve of the cat reveals that this nerve contains an abundance of myelinated fibres which can be divided in two main groups: a) fibres with a diameter of from 4 to  $7 \mu$ ; and b) fibres of a diameter of from 1 to  $3 \mu$ . The preganglionic efferent fibres belong to the latter group, while the group of thicker fibres obviously consists of afferent fibres conveying impulses from the Pacinian corpuscles in the mesentery and noxious impulses from the abdominal region. Judging from the amplitude of the action-potentials from single fibres set up by mechanical stimuli applied to the Pacinian corpuscles of the mesentery, the largest fibres of the splanchnic nerve must be considered to convey impulses from these receptors. Noxious stimuli most probably set up impulses in myelinated fibres of various sizes ranging from 6 to  $1 \mu$  as well as in unmyelinated fibres. According to the nomenclature formerly used by one of us (ZOTTERMAN 1939), impulses from the Pacinian corpuscles are conveyed in  $\beta$ -fibres, while pain is conducted by fibres belonging to the  $\delta^1$ - and  $\delta^2$ -groups as well as to class C.

As has already been observed by ADRIAN (1930), the spontaneous impulses set up at the cut end of nerve fibres disappear when the thick sheath is pulled off. He also states that when the sheath is very thin (which is the case with the smallest nerves) the action is as a rule very slight. We have been able to confirm these observations in numerous cutaneous and other nerve preparations.

Our first impression of this continuous flow of action-potentials in the splanchnic nerve was that they should be looked upon as injury potentials set up at the cut peripheral end of the nerve. The direction of the monophasic response, however, indicated that the impulses were conducted in a centrifugal direction. The nerve was prepared and the electric activity was recorded in the same way as was described in our previous paper (GERNANDT, LILJESTRAND and ZOTTERMAN 1946).

By moving the electrodes 20—30 mm along the exposed nerve, which is suspended in the air, we were able to convince ourselves that the discharge was not set up in that part of the nerve, but more centrally. In another preparation which showed the same phenomenon we were able to disclose the fact that the discharge originated from the part of the nerve where it left the tissues. The sheath was drawn off to this point. This procedure of drawing off the

sheath from the cut peripheral end in a central direction is very easy for the first 10 to 20 mm. After that we gradually had to pull harder, and it seems very likely that the nerve may be somewhat damaged just at the point where it leaves the surrounding tissue. In following preparations which displayed this continuous discharge of fairly large spikes, we were in any case able to demonstrate that the discharge took place from that part of the nerve which was covered by the sheath and which was situated very close to the point where the nerve entered the tissues. After having dissected out the nerve further centrally, both electrodes could be placed upon the part of nerve which was covered by the sheath. Now the recorded direction of the spikes showed that in this part of the nerve they were conducted in a centripetal direction.

It was thus absolutely proved that the impulses were not set up in the naked and exposed part of the nerve. We stress this circumstance because such a finding would be incompatible with the fact that the continuous discharge can be modulated by changes in the respiration of the cat. Thus we first observed that the discharge invariably diminished in strength when the tracheal tube was clamped. A further analysis revealed the fact that the spontaneous discharge was very highly augmented by hyperventilation, while hypoventilation diminished the activity. As is shown in fig. 1, the spontaneous discharge of large spikes can be very highly reduced by stopping the artificial respiration for 30 seconds (fig. 1 c). In this case the great reduction in the discharge of large spikes reveals the activity of the small preganglionic fibres. 10 to 15 seconds after artificial respiration had been started again this efferent discharge was quite abolished, as has been previously reported (GERNANDT, LILJESTRAND and ZOTTERMAN 1946). The antidromic afferent discharge gradually returns, however, and generally reaches its previous level in about one minute (see fig. 1 d and e).

An interesting point was now to see how the antidromic discharge reacted to anoxemia and to hypercapnia. The cat was thus ventilated with 5.9 % oxygen in nitrogen. This gave a very definite increase in the discharge (see fig. 2 b), while ventilation with 13 % CO<sub>2</sub> in oxygen quickly reduced the activity and usually abolished all activity of this kind within 10 to 20 seconds (see fig. 2 c). The irregularly shaped potentials seen in fig. 2 c are due to the efferent preganglionic fibres.

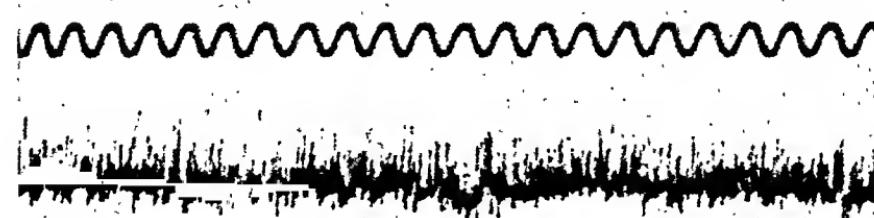
*A**B**C**D**E*

Fig. 1. Records showing the continuous discharge of antidromic afferent impulses in the splanchnic nerve of the cat. Chloralose anaesthesia 0.05 g per kg body-weight.

*A.* Standard ventilation of the lungs; *B.* after one minute of over-ventilation; *C.* artificial respiration stopped for 30 seconds; *D.* Artificial respiration resumed for 10 seconds; *E* d.o. 90 seconds later. Time marker 50 cycles per second.

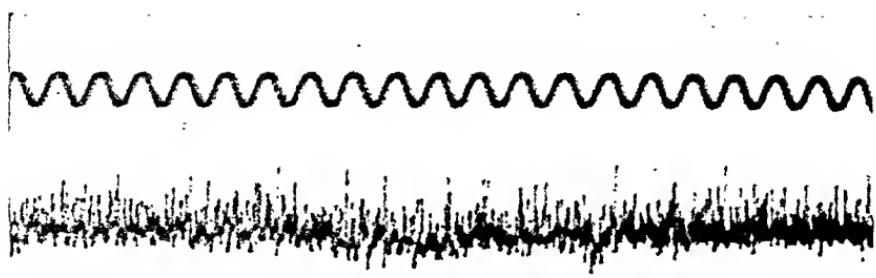
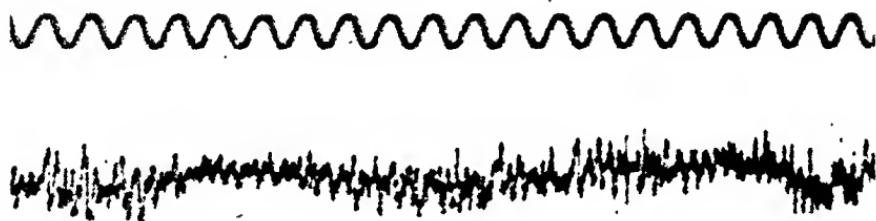
*A**B**C*

Fig. 2. Injury potentials from the splanchnic nerve of the cat.

*A.* Under standard ventilation with air; *B.* with 5.9 % O<sub>2</sub> in N<sub>2</sub>; *C.* with 13 % CO<sub>2</sub>, which inhibits the discharge of large spikes and thus discloses the efferent volleys of impulses in the preganglionic fibres (Class B fibres).

For the further analysis we considered it worth while to test the effect of eliminating the effect of the carotid sinus and the depressor activity by cutting Hering's nerve and vagus on both sides. This procedure had no influence upon the large spike activity under standard ventilation, as will be seen in fig. 3 a. This record shows that the preganglionic fibre activity is definitely augmented, owing to the elimination of the inhibitory afferent volleys from Hering's nerves and the depressor nerves. Ventilation of the lungs with 13 % CO<sub>2</sub> in O<sub>2</sub> has the same action as previously upon the discharge of the large spikes, which is reduced almost to nil, while the effect upon the preganglionic fibres is now definitely increased (fig. 3 b). The effect of 5.9 %

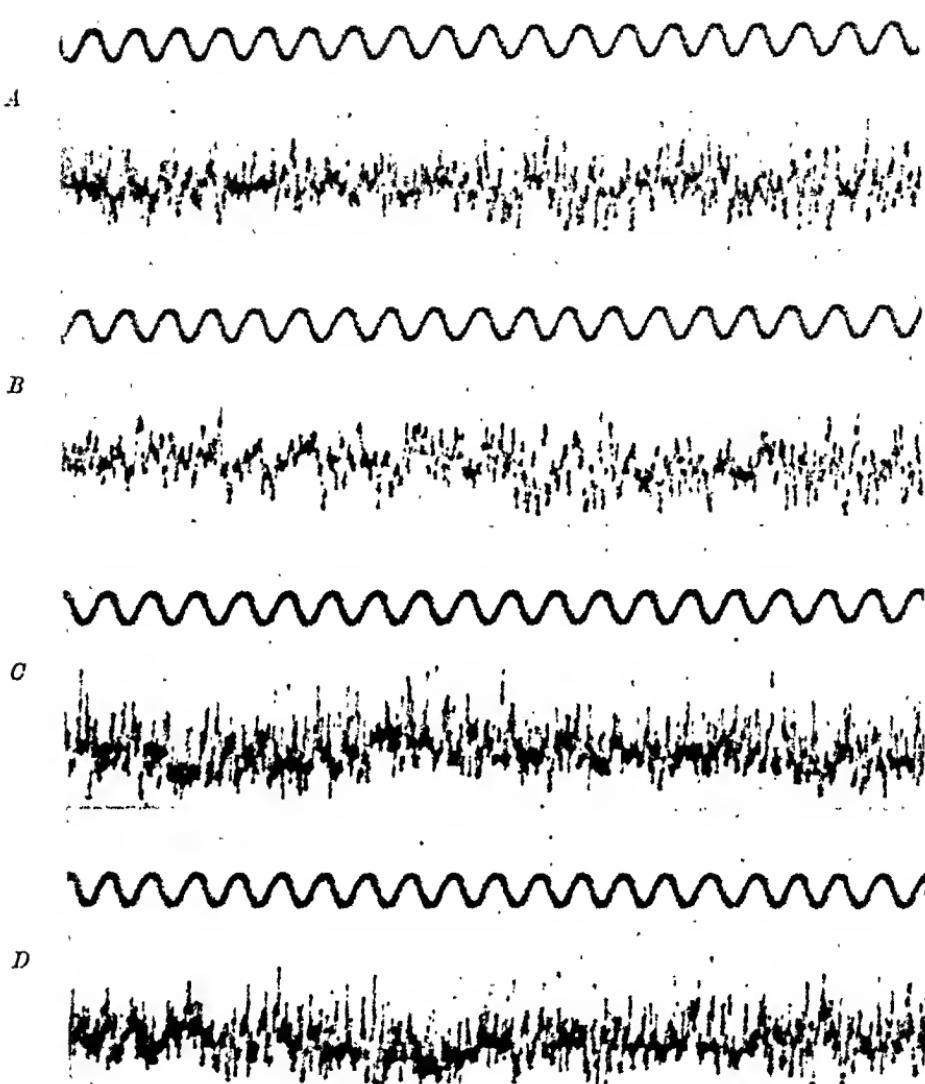


Fig. 3. Records from the splanchnic nerve of the cat; same preparation as in figs. 1 and 2. Hering's nerve and vagus cut on both sides, which augments the B-fibre activity.

A. during standard ventilation; B. artificial ventilation stopped for 30 seconds; C. standard ventilation again; D. artificial ventilation with 5.9 % O<sub>2</sub> in N<sub>2</sub>.

oxygen in nitrogen, however, had now disappeared. This can be explained in the following way. As long as the chemoceptors are intact the low oxygen pressure causes an increased ventilation which results in a hypocapnia. After the cutting of all chemo-

ceptive nerves low oxygen pressure does not induce any increase in the ventilation, thus keeping up the CO<sub>2</sub> tension as before (see fig. 3 C).

### Experiment on Man.

The effect of respiratory changes upon the spontaneous discharge of afferent injury potentials in the cat induced us to study similar phenomena in man. A similar spontaneous injury discharge can easily be produced by pressure and asphyxia to the nerves of the arm by inflating a cuff above the elbow and keeping the pressure above the systolic blood pressure for a period of 5 to 10 minutes. The sensation of tingling following upon the release of pressure has been thoroughly described by LEWIS, PICKERING and ROTSCCHILD (1931) and ZOTTERMAN (1933). KUGELBERG (1944) has shown that the rheobase of the motor nerves of the arm was essentially lower in tetany and during hyperventilation of the subject. He has also shown that the slope of accommodation increases progressively as the ischemia goes on. And finally, he has shown that after the release of the blood-flow to the arm there is a sudden heavy fall in the slope of accommodation, and for a period of 2 to 7 minutes after the release there is a break-down of accommodation. As far as we can see, the duration of this break-down of accommodation coincides very well with the temporary course of the post-ischemic pricking sensations.

*Production of post-ischemic tingling.* The intensity of tingling depends upon the time the arm has been compressed. As shown by ZOTTERMAN (1933), the minimum compression time varies individually but does not seem to extend over 7 minutes. For this reason we have in our experiments chosen 7 minutes as the standard time of compression. The cuff used was 12 cm broad and was placed immediately above the elbow. It was distended to 150 mm Hg. After release there is always a feeling of a wave of warmness and heat in the skin below the cuff and then after a silent period come the pricking sensations, which start in one or more finger-tips, usually the thumb and index finger. The interval between the moment of decompression and the start of the pricking sensations varies individually and also from time to time, as is shown in table 1, which gives the latency, the maximal subjective strength and the duration of the tingling in two series of experiments upon four healthy subjects. The subjects reported the start and end of the tingling and gave the course of increase

and decline of the subjective sensations. The intensity of the sensations was given in seven degrees, described as a gliding subjective scale of sensations from the faintest pricking to a very painful sensation, which is clearly reflected in the behaviour of the subject.

When the subjects breathed air normally the latencies of tingling varied from 55 to 80 seconds and the duration of the tingling varied from 2 to 3 minutes. The maximum subjective strength of tingling varied from 2 to 3 degrees, which means that the tingling at its maximum was quite distinct but not in any way disagreeable.

As will be seen from table 1, the strong over-ventilation which commences at the moment of decompression brings about a very great change in the picture. The latency was now as a rule distinctly shortened, in one case to less than one half. The tingling rose steeply to a very high degree and was reported as very painful and disagreeable. In some subjects the over-ventilation led to wide-spread symptoms. As long as the subject was over-ventilated, the highly painful tingling went on without any sign of decrease. For this reason the experiment was discontinued after 6 minutes.

In the light of our observations on the spontaneous discharge of afferent impulses in the cat's splanchnic nerve, we expected that the phenomenon would be changed in the opposite direction by inhalation of carbon dioxide. This expectation was confirmed. Breathing from a Douglas bag a gas mixture consisting of 7.1 % CO<sub>2</sub> in air lengthened the latency of tingling. In one case no tingling appeared. The maximum subjective strength as well as the duration of the tingling was very markedly reduced. An increase to 10.4 % of the CO<sub>2</sub>-content of the inhaled air reduced the tingling still more. This percentage of CO<sub>2</sub> was of course close to the upper concentration which can be endured for a period of up to 5 minutes. The subjects now reported that the tingling, which started after a still longer latency than before, was so faint that they would scarcely have observed it if their attention had not been especially directed thereto. The pricking was felt only in one fingertip and on one spot only, and it was experienced as a very faint pricking occurring with silent periods up to 10 seconds.

An attempt to illustrate the strength and the course of the post-ischemic tingling under the various conditions described above has been made in fig. 4, the diagram of which is constructed upon the data obtained from one of the subjects. (O. N.).

Table I.

Subject	Normal breathing			Forced breathing of air			7.1 % CO <sub>2</sub> in air			10.4 % CO <sub>2</sub> in air		
	Latency of tingling sec.	Max. strength of tingling	Duration minutes									
A. E.	60	3	2	50	6	> 6	70	2	1.83	80	< 1/3	0.7
,	75	2	2.5	50	6	> 6	∞	0	0	∞	0	0
H. A.	80	2	2	55	7	> 6	85	2	2	105	< 1/2	0.5
,	68	2	2	62	7	> 6	80	1	2	75	< 1/2	0.8
T. S.	70	3	2.8	30	5	> 6	∞	0	0	∞	0	0
,	65	3	2.7	50	4	> 6	85	2	2	105	< 1/2	0.5
O. N.	61	3	2.3	45	7	> 6	80	1	2	75	< 1/2	0.8
,	55	3	3	58	7	> 6	∞	0	0	∞	0	0

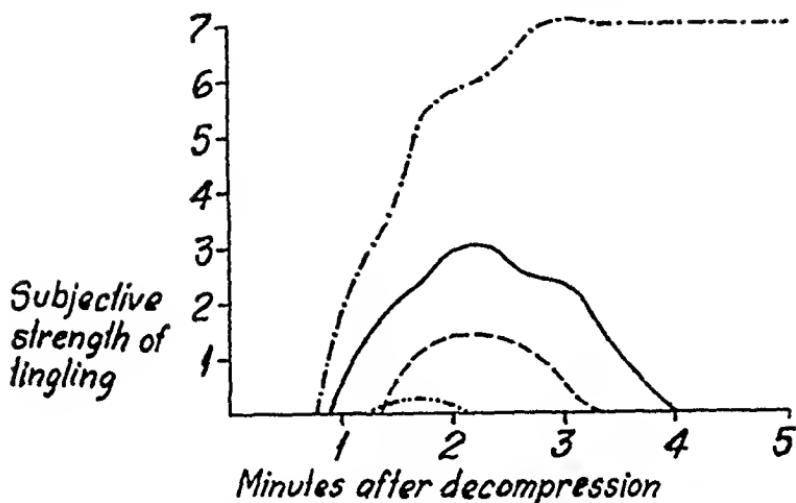


Fig. 4. Diagram showing the latency and course of tingling after 7 minutes of compression of the arm above the elbow.

— normal breathing of air; - - - - forced breathing of air;  
- - - breathing of 7.1 % CO<sub>2</sub> in air; ····· breathing of 10.4 % CO<sub>2</sub> in air.

### Discussion.

It has previously been suggested (ZOTTERMAN 1933) that a lowering of the concentration of metabolites in the tissues plays a decisive rôle in the establishment of postischemic tingling. The long latency for the tingling indicates that it is not elicited by the mechanical action of the blood-flow forcing its way through the previously compressed part, but shows that the tingling does not set in until after the blood supply has been restored for half a minute or more. The reactive hyperemia developed in the decompressed region no doubt is followed by a very ample blood-flow, which quickly changes the reactive properties of the larger myelinated nerve fibres in the direction shown by KUGELBERG (1944). It is thus not surprising to find that changes in the CO<sub>2</sub>-tension of the blood will produce very definite quantitative changes in the postischemic tingling sensation. These changes in the tingling are obviously quite comparable to the spontaneous discharge of injury action-potentials from afferent fibres observed in the splanchnic nerve of the cat. Both phenomena are reduced in strength or inhibited by an excess of carbon dioxide in the circulating blood; on the other hand, the activity is strengthened or initiated by a reduction of the carbon-dioxide-tension of the blood.

The changes in the carbon-dioxide-tension of the blood in these experiments undoubtedly brought about very definite changes in the pH of the blood and the tissues. Thus, breathing of 10 per cent carbon dioxide will cause a definite increase in the amount of ionized calcium in the tissues, while an increase of the pH caused by strong over-ventilation of the lungs brings about a lowering of the Ca-ion concentration.

LEHMANN (1937) showed that readmission of oxygen to previously asphyxiated peripheral mammalian nerves caused within 1 to 2 minutes a rapid fall in the threshold to a normal value. The threshold then rose for a second time and remained high for about 50 minutes. This latter change in the excitability is very similar to that brought about by a change of the pH from 8 to 7.4 in the surrounding solution. He also showed a further important fact, *i. e.* that during the period of low thresholds the afterpotentials are decreased, and the nerve becomes spontaneously active as it does in a state of low calcium.

Another point which is of interest in this connection is that strong tactile stimuli to the finger-tips applied when the pricking paresthesia is at its height cause a very sharp and severe pain. It has been suggested by ZOTTERMAN (1933) that this phenomenon is most probably caused by the pain fibres in the previously compressed part of the nerve being stimulated secondarily by the action-potentials of adjacent tactile fibres. GRANIT, LEKSELL and SKOGLUND (1944) have recently demonstrated that an interaction of different fibres actually does take place in injured or compressed regions of a nerve. Thus the increase of the pricking paresthesia induced by peripheral tactile stimuli can be interpreted in the light of these facts. In a second paper GRANIT and SKOGLUND (1945) state that the "artificial synapse" formed by the cut end of a mammalian nerve is best demonstrated in decerebrated cats which have not lost too much blood and in cats under chloralose narcosis, while cats under "dial" were too deeply narcotized. We would suggest that this difference is not effected by any direct action of the narcotics upon the peripheral nerve, but depends upon differences in the ventilation of the lungs.

### Summary.

1. Injury potentials set up in the afferent fibres of the splanchnic nerve of the cat have been shown to respond in a regular manner to changes in the carbon-dioxide-tension of the blood. Artificial over-ventilation with air as well as the increased ventilation ensuing from breathing gas mixtures low in oxygen produce a very marked increase of the injury potentials, while a rise in the carbon-dioxide-tension of the blood inhibits the discharge.

2. In full accordance with the above phenomena it was found that the pricking paresthesias following upon the release of the bloodstream to the arm after a previous period of asphyxiation can be modulated in a corresponding way by changing the carbon-dioxide-tension of the subject; hypocapnia increasing them and hypercapnia causing a reduction or abolition of the sensations experienced.

3. These findings are discussed and related to the theory advanced by LEHMANN to the effect that the variations in the irritability of mammalian nerve fibres produced by changes in the  $C_H$  of the tissue are due to changes in the amount of ionized calcium.

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# Methods for Continuous Tissue Culture as Applied to Bone Marrow.

By

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Experiments on the development of cells in vitro can provide a certain amount of information about the development of various tissues, their metabolic processes etc.

In hematological work tissue culture methods have up to now only been used for isolated observations in connection with theoretical considerations, as for instance the denucleation of erythrocytes, the formation of thrombocytes etc., but it ought to be possible to utilize them to approach the solution of numerous hematological problems, and I have attempted to work out suitable methods for such purposes.

I shall in the following briefly describe an apparatus which has been successfully applied for cultivation of bone marrow cells over long periods of time and which will, I believe, be applicable also to other branches of tissue cultivation.

In 1936 (at about the time when the first attempts on cultivation of human bone marrow were made) Osgood published a method for the culture and study of liquid bone marrow. His intention was to provide conditions for the bone marrow suspension approaching the normal as closely as possible viz., the apparatus would function as lung, kidney and organ of circulation for the marrow, and at the same time the construction would allow sterile samples of the suspension to be taken at any time during the progress of an experiment. Simultaneously Osgood made a number of suggestions concerning inquiries into conditions of cultivation and development of cells for which the apparatus would appear to be suitable in view of the physiological conditions aimed at. In spite of these possibilities Osgood and

his coworkers replaced the method by a simpler one after half a year's trial. In this new method the close approach to physiological conditions was abandoned and a constant composition was not maintained neither for the ventilating air current nor for the culture medium.

Although OSGOOD's second method would be easier to work with, I considered the physiological conditions so important that I decided to base my construction as far as possible on the principles of Osgood's first method. I found it necessary to work out two constructions, one for the prolonged experimentation on fairly large samples and another, embodying as far as possible the same principles, but allowing continuous microscopic observation of the cells under culture.

### I. A method for the cultivation of large samples.<sup>1</sup>

After a number of preliminary experiments with an apparatus very similar to Osgood's first construction I adopted a somewhat modified arrangement which, while still fulfilling the primary conditions, was in many ways easier to work with.

For practical reasons I had four identical apparatus constructed and all of them mounted in the same thermostatic water bath, a point of considerable importance for quantitative comparative work.

As stated above normal physiological conditions were the main consideration, and it is possible to regard the apparatus as made up in sections representing respectively the supply of nourishment, the respiration, the circulation and the excretion of waste products. These sections are to a certain extent separated by membranes permeable to gases and crystalloids and corresponding more or less to the vascular endothelium and the kidney glomerular membrane. In the original Osgood apparatus "parlodion" was used for these membranes, but owing to the war this substance was unobtainable. I first prepared membranes from collodium, which were difficult to get uniform, and later I used the ultrafiltration membranes described by REHBERG (1943) and prepared by the Copenhagen firm "Kapeello".<sup>2</sup> These were made

<sup>1</sup> The macro-apparatus was made by *H. Struers chemiske Laboratorium*, Copenhagen, who will be ready to supply it under my supervision. I want to thank Mr. RASMUSSEN, cand. pharm., of the firm for his helpfulness during the construction.

<sup>2</sup> My thanks are due Mr. Hawlik, Civil Engineer of this firm for his kind assistance in providing these necessary membranes.

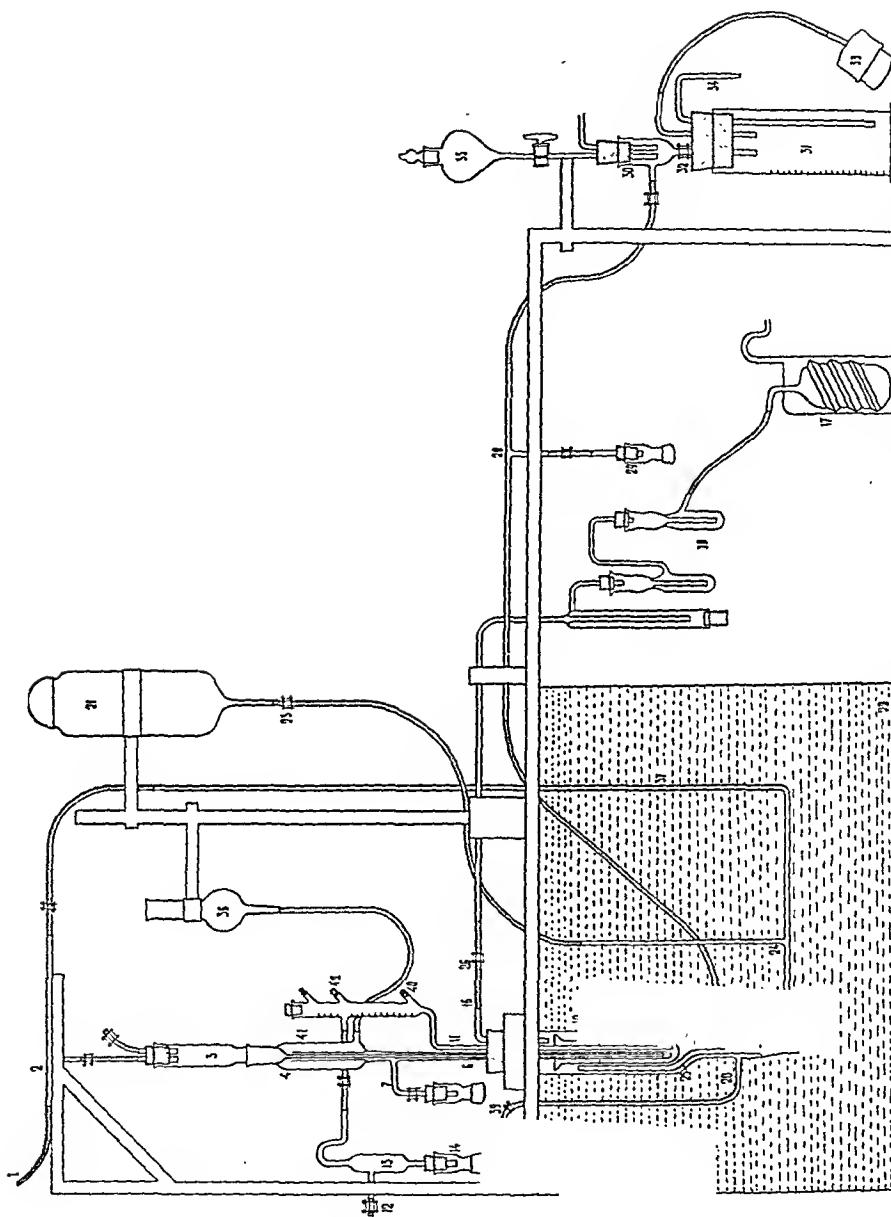


Fig. 1. Diagram of the apparatus for the cultivation of large samples of bone-marrow cells in suspension.

for me in two sizes, viz. diameter 18 mm, length 110 mm, and diameter 9 mm, length 50 mm. Both are practically impermeable to colloids.

I shall now describe in detail the apparatus as in use during an experiment, referring by numbers to the figures 1 and 2.

### The provision of nutriment.

In large scale tissue culture work it is important to provide a continuous supply of food for the culture. This is done in this apparatus by a constant flow of the appropriate solution through the internal membrane (5, fig. 2). The solution enters through the tube in the top left corner (1, 1) passes through the T-tube (2, 1), the drip vessel (3, 1), internal diameter: 18 mm, and the capillary tube (4, 1 and 2), internal diameter: 1, 2 mm, which opens near the bottom of the 9 mm membrane (5, 2). From here the fluid rises through the membrane and the tube (6, 2), internal diameter: 7 mm, to which the membrane is attached and leaves through the side tube (7, 1), where it can be collected in a bottle (not shown). The rate of the flow of the fluid was in my investigations 80—90 ml/hour.

The nutritive substances diffuse out through the membrane (5, 2) thereby supplying the culture in the membrane (8, 2) and the concentration here will remain approximately constant when the supply is large compared with the amounts used up.

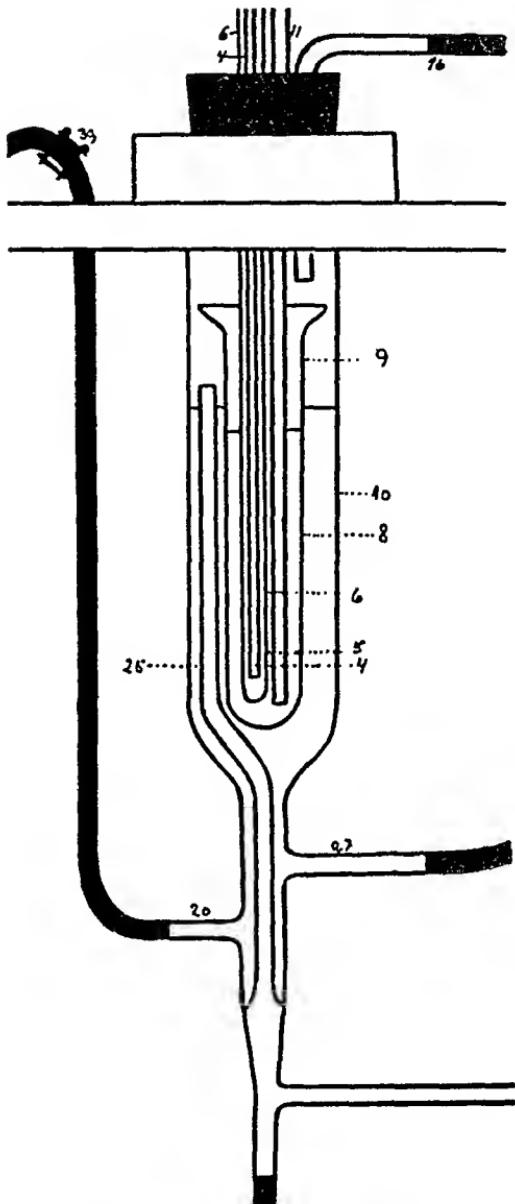


Fig. 2. A fragment of the diagram fig. 1 showing the membranes placed in the apparatus.

The membrane (8, 2) containing the cell suspension — bone marrow cells suspended in Ringer-solution — under culture is fastened to the glass funnel (9, 2) resting on three projections inward from the outer vessel (10, 1, 2).

### Aeration and determination of respiratory metabolism.

The cell suspension (in 8, 2) is kept in continuous movement by a current of air (or other gas mixture) through the tube (11, 1, 2) and thereby prevented from sedimentation. The rate of the air flow was 100 ml/hour.

The air which is made CO<sub>2</sub>-free previously is let into the apparatus through the tube (12, 1) and passes through the vessel (13, 1) for condensing water vapour. (14, 1) is an arrangement for taking of gas samples for analysis through the rubber by means of a cannula and a common air recipient. Passing on to the vessel (15, 1) inserted for the sampling of the cell suspension (see later) the air is filtered through cotton wool and passes through the tube (11, 1, 2) to the membrane (8, 2) in which some O<sub>2</sub> will be absorbed and CO<sub>2</sub> added. The (expired) air passes through the tube (16, 1) first to the glass vessels (18, 1) for the condensation of water vapour and then to the CO<sub>2</sub>-absorber (17, 1). In ordinary experiments the absorber contains lime-water [Ca(OH)<sub>2</sub>] stained with phenolphthalein to estimate the CO<sub>2</sub> production, but in special respiratory experiments the absorber is replaced by an almost vertical tube (75°) length 750 mm, diameter 12 mm containing Ba(OH)<sub>2</sub> and the air is admitted from below through a capillary tube placed in a rubber stopper. This arrangement was very effective for absorbing and titrating small quantities of CO<sub>2</sub>. The Ba(OH)<sub>2</sub> was emptied, by removing the rubber stopper, down in a small flask and titrated (WINKLER 1931). When determinations of CO<sub>2</sub>-production were made one of the four apparatuses was used as a control and the membrane (8, 2) filled up with Ringer-solution without cells. Some CO<sub>2</sub> will diffuse out through the "glomerular" membrane (8, 2) into the outer vessel (10, 1, 2) and, to secure this, "inspired" air is added through (19 and 20, 1), bubbles through the solution for removal of waste products and leaves the air from the cell suspension.

### Removal of waste products.

For technical reasons it was not found practical to have a continuous flow of solution along the outside of the membrane (8, 2). A large volume of solution ( $15 \times$  the cell suspension) was therefore placed between the membrane (8, 1, 2) and the outer vessel (10, 1, 2) and changed at intervals which had to be fairly short (30 min.). The change is performed as follows: The solution (in 10, 1, 2) is removed by clamping (26, 1) on the expired air tube. This raises pressure and drives out the fluid through (27, 1, 2), (28, 1) provided with an arrangement for sampling (29, 1) to the funnel (30, 1) and the cylinder (31, 1), where it is collected. The funnel (30, 1) has a reservoir with alcoholic neutral red for determination of the pH, when desired. The cylinder can be emptied out after closure of (32, 1) by means of the pump (33, 1).

Fresh solution (usually suitably buffered Ringer) of body temperature is contained in the reservoir (21, 1). When the clip (23, 1) is opened the solution will flow through (24, 1) and (25, 1, 2) and fill up the chamber (10, 1, 2), emptied just previously.

It is of course necessary to work in sterile conditions. Since the complicated apparatus would be difficult to sterilize completely I preferred to work antiseptically by adding "solbrol" (methyli-para-oxybenzoas) in a concentration of 1 % to all solutions. This proved harmless for the cells studied.

Before use the membranes were kept in water with added "solbrol", the Ringer solution used for cell suspension and waste elimination was sterilized by boiling and "solbrol" was added. The bone marrow was sucked out through a sterile cannula into a sterile syringe of 20 ml capacity. After this the cells (0.5 ml) were suspended in 25 ml Ringer solution. The technique used for getting the bone marrow cells is to be published in detail elsewhere.

In the experiments so far made infections have occurred only very rarely, but then of course spoiled the experiment.

An experiment is started as follows: The membranes are mounted in the apparatus and tested for tightness by means of Ringer solution, especially at their connection with the respective glass tubes brought about by 5 mm broad rubber bands of 1 mm thickness, the diameters are a little smaller than the respective glass tubes. The whole apparatus is put together and air sent through

for 15 minutes during which the solution in the outer chamber is changed 3 to 4 times. Next the flow of air is stopped by closing the clips (38, 1, 39, 1 and 26, 1), and the Ringer solution in the membrane (8, 1, 2) is sucked up in the tube (15, 1) by means of a cannula introduced through the vacuum-rubber tube (42, 1) and a syringe, capacity 30 ml. Then another cannula is pierced through the vacuum-rubber (40, 1) and the Ringer solution is sucked out. After removing the cannula in the rubber tube (40, 1), the sterile bone marrow suspension is injected into the tube (15, 1) through the cannula in the rubber (42, 1) and the cannula is removed. The volume of the bone marrow is then read by means of the graduation in the tube (15, 1) diam. 8 mm and graduated in 0.5 ml.

Then the clips (26, 1 and 38, 1) are opened and the air flow will press down the bone marrow suspension into the membrane (8, 2). When the suspension is down in the membrane the clip (39, 1) is opened.

When samples of the suspension are to be taken during the experiment it is sucked up into (15, 1) and the volume is measured, then the necessary quantity is drawn out by means of a small syringe — 2 ml — from (40, 1).

In cases in which a flow of nutritive solution is not desired, the membrane (5, 2) may be compressed and to avoid this water may be introduced into the chamber (41, 1) opening into the tube (6, 1) from the reservoir (36, 1) and thus raise the pressure.

In some cases it may be practical to arrange a flow of the nutritive solution also through the compartment (10, 1, 2) and to effect this the second branch of the T-tube (2, 1) is connected through the tube (37, 1) also with (10, 1, 2) and the solution in the chamber (10, 1, 2) is changed as described.

The apparatus as here described may at first sight appear more complicated than OSGOOD's first model, but in return for this the life conditions of the cells are sufficiently good to maintain the normal structure of the blood corpuscles and therefore, probably, also their functions. It should be mentioned that while in the first experiments, in an apparatus copied from OSGOOD, destructive changes were often observed in the erythrocytes, even a crenation was only very rarely seen in the apparatus described.

The apparatus as described will render possible the study of a number of hematological problems including metabolism de-

The whole is placed on a heating table under the microscope. The thickness 0.14—0.17 mm, held down by the two clips (10, 3, 4), take the cell suspension and finally an ordinary coverslip (9, 4) to 0.17 mm) coverslip with a 2 mm hole in the center (7—8, 4) to is a thin plane colloidal membrane (6, 4) another thin (0.14— and carrying off the solution. Above the coverslip (2, 4) again side trenches, serving respectively for feeding nutritive solution on. Then two canulas are embedded (4 and 5, 3) in the two last ing — provided with a central hole of 2 mm (3, 4) is now sealed bear a coverslip (2, 4) — the usual one for blood corpuscles counts was placed to level the counting chamber. On the top of the chamber was placed to a small amount of the same substance (1, 3) with asphalt and a small amount were filled up employed. As shown in fig. 3 the two side trenches were filled up emulsions. As a basis for this a Bürker-Türk counting chamber was Brock and Jülicher, Openhausen, according to my instructions. It is often important to be able to follow the changes in a particular cell, and to enable one to do this, an apparatus which could be placed under the microscope, was made for me by the firm Bröck and Jülicher, Goppenhausen, Germany to my instructions. It is studied only on representative samples secured at intervals. In the apparatus as described above the changes taking place can be seen only on the culture of the culture.

## II. A micro-method for the bone marrow culture.

One modification now to be described allows an uninterrupted microscopic study of the culture.

improved so as to approach more closely the physiologically normal binary. It can be suitably modified for any purpose and also conditions within the organism.

The technique as here described is to be considered as preliminary.

In my experiments I have kept the culture for 24 hours, but

Osgood reports that he has made investigations on cultures 72 hours old. In the effect of bactericidal substances on bacterial cultures on the effect of bone marrow and of isolated groups of cells, studies vitamins, hormones etc., determinations of the respiratory metabolism, necessary for the cell proliferation and development; amino acids, CO<sub>2</sub>, tension for the cell culture, determination of the substances viz. determinations of the optimal temperature, pH, oxygen and CO<sub>2</sub>, which summarize some of the possibilities opened up,

terminations on the bone marrow as a whole or on isolated groups of cells.

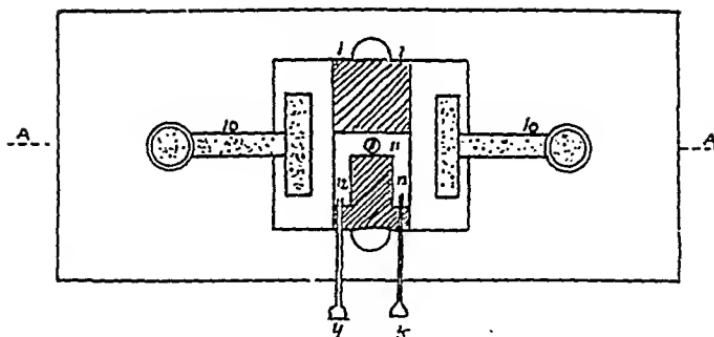
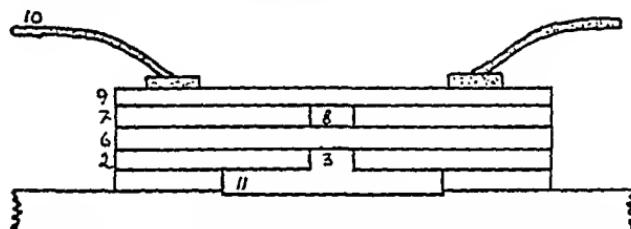


Fig. 3. Diagram of the apparatus for the cultivation of isolated cells of the bone-marrow.



An enlargement of the central part of the apparatus shown in Fig. 3.

Nutritive solution, suitably heated and saturated with oxygen, flows from a DEWAR vessel through the apparatus at a rate of 80 ccm/hour (to keep up the temperature).

This apparatus may be constructed a little cheaper when not using a Bürcker-Türck counting chamber but by using a 3 mm thick object slide as basis and then mount by means of heated Canada balsam some glass pieces to form the lower drain made by the trenches of the counting chamber.

The observation of the cells is rather difficult since they must remain unstained and only differences in size and shape can be observed. Usually, however, the nucleus appears a shade lighter than the rest of the cell content. The use of Zeiss' "Phasenkontrast-Einrichtung" kindly lent by the BROCH and MICHELSSEN somewhat facilitated the observations.

The two types of apparatus described above have been used since May 1944 and proved satisfactory although requiring a certain amount of training. They have been used so far only for investigations concerning the erythropoiesis and the main results, which will be published in detail elsewhere, are as follows: A new formation of red cells takes place even with Ringer solution as the feeding liquid. The numbers of mitoses observable in

differential counts appear able to account for only 1 per cent of this new formation which must therefore take place by a different mechanism. When liver extract is added to the feeding fluid the number of erythrocytes formed is increased in a certain relation to the concentration of the extract, but without any increase in the number of mitoses. When the mitotic activity is completely paralyzed by the addition of colchicine to the feeding liquid — as well as to the cell suspension — new red cells are produced at the same rate as before.

It is to be concluded from this study that erythropoiesis takes place in two separate stages. In the first stage normoblasts are formed by mitotic cell division, while in the second immature and small red corpuscles are formed from the normoblasts by segregation of protoplasma pseudopodia. The normoblasts finally die after giving off a certain number (about 100) of corpuscles.

LISA BOSTRÖM (1940) suggested a formation of erythrocytes from normoblasts by segregation of pseudopodia without any nucleus, and by means of the micromethod described above I have been able to observe the process as follows: the initially spherical normoblast gradually (10—15 min.) became oval when fed with liver extract, most of the protoplasm flowed toward one end opposite the nucleus and suddenly a drop of protoplasm was cut off and a small erythrocyte formed. This small erythrocyte slowly grew in size and in one hour or so it got nearly the same shape and size as the normal erythrocytes in the suspension.

The constructions of the apparatus described above were made possible by a grant from the *Carlsberg Foundation* and the work with them supported by a grant from the *King Christian X Foundation*.

My sincere thanks are due to both these foundations. I want to thank also the firm Medicinalco Ltd for the opportunity to carry on this investigation in the biological laboratory.

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From the State Pharmaceutical Laboratory, Stockholm,

## The Influence of Different Temperatures on the Action of Drugs on Autonomic Effector Cells.

By

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Fundamental investigations by ELLIOT, LOEWI, DALE, CANNON, and others have given us a considerably greater knowledge than before regarding the physiology of the autonomic nervous system. In connection with DALE's theory of the chemical transmission of nerve impulses, we have obtained a better explanation than heretofore of the mechanism and site of action of autonomic drugs, even if many problems of considerable importance in this field are still unsolved.

Among methods for the interpretation of the pharmacological action of drugs, *in vitro* experiments on isolated surviving organs are employed. In such experiments it seems by no means inconceivable that certain structures of the effector cells are more sensitive to temperature changes than others. In that case, raising or lowering of the temperature as compared with the normal, in different ways as regards drugs with different sites of action, might affect the response of the effector organ.

The author of this paper has therefore studied the problem whether the temperature could be made the basis of a test which, in certain cases, might contribute to greater knowledge of the mechanism of action and classification of autonomomimetic drugs, or confirm results obtained with other methods.

To judge by the literature, this problem does not appear to have been previously subjected to investigation. On the other

hand, isolated studies have been published, in another connection, regarding the bearing of the temperature on the action of certain drugs on surviving organs.

Thus, LAUBENDER and his co-workers, in a number of publications, have reported their observations on the action of some drugs (histamine, acetylcholine, orastin and coniin) on smooth and striated muscles, with special regard to the relation of the effects to the concentration of the drug and the reaction temperature. Their studies were made on the gastrocnemius of the frog at temperatures ranging between 5 and 20° C and on the uterus of guinea pigs between 18° and 37°. As a rule, a stronger effect was obtained with rising temperature except for acetylcholine, where the intestine showed a maximum effect at about 28°.

RENTZHOG (1930), in a study of the effect of epinephrine on isolated surviving small intestine of rabbit at varying temperatures, found a maximum effect at about 28°. A similar observation was made by BLASCHKO and SCHLOSSMANN (1938), who observed that the small intestine of rabbit was most sensitive to epinephrine at about 30°.

ZADINA (1938) observed that histamine gave the strongest effect on the isolated small intestine of guinea pigs at temperatures below 37°. EMMELIN, KAHLSON and WICKSELL (1941), in regard to the intestine of guinea pigs, found a temperature of 32° in the bath fluid to be the most favourable in the estimation of histamine in the plasma.

## I. Methods.

The author's experiments were performed on the isolated surviving small intestine of rabbits according to the technique of MAGNUS. The intestinal segments were suspended at 38° C in Thyrode solution, consisting of NaCl 0.8 %, KCl 0.02 %, CaCl<sub>2</sub> 0.01 %, MgCl<sub>2</sub> 0.01 %, Na<sub>2</sub>HPO<sub>4</sub> 0.005 % and NaHCO<sub>3</sub> 0.1 %. Through the solution was bubbled a mixture of 95 % O<sub>2</sub> and 5 % CO<sub>2</sub>.

In the following list of investigated drugs the number of micrograms of the respective drugs per c.c. fluid in the bath ( $\gamma/ml$ ), that was as a rule found to be the most suitable dosage for the experiment, is given in brackets.

Acetylcholine hydrochloride (0.01), acetyl- $\beta$ -methylcholine hydrochloride = mecholyl (0.01), acetyl- $\beta$ -methylcholine bromide = mecholyl-bromide (0.01), carbaminoylecholine hydrochloride = doryl (0.01), pilocarpine hydrochloride (0.3), physostigmine (eserine) salicylate (0.03), prostigmine methylsulphate (0.1).

1-epinephrine hydrochloride (0.01), 1-corbasil hydrochloride (0.03), d-corbasil base (0.9), dl-arterenol hydrochloride (0.04), dl-adrenalone hydrochloride (3), dl-ephedrine hydrochloride (9), l-adrianol hydrochloride (0.6), l-sympatol hydrochloride (0.3), dl-benzedrine sulphate (1.7), dl-veritol sulphate (2.9).

Atropine sulphate (0.06), ergotamine tartrate (0.1), substance P, EULER and GADDUM (1—6 E.), nicotine sulphate (2.8), histamine (0.9), barium hydrochloride (3).

The changes in temperature were effected in the following manner. The result of such a dosage of the respective drug as gave a moderate effect at a temperature of 38° C., was first observed. Afterwards the effect of the same dosage at a somewhat lower or higher temperature (some of the temperatures ranging between 38° and 18° and, respectively, between 38° and 40° in the bath fluid). The experiment was then repeated at the original temperature of 38°. This procedure was repeated the largest possible number of times (*e. g.* 38°—24°—38°—24°—38°) on each piece of gut. At each temperature at least two effects of the drug in question were studied before the temperature was changed. After each change of temperature 10 minutes as a rule were allowed to elapse before a drug was added to the bath.

The preliminary investigations showed that temperature variations between 38° and the lower degrees of temperature stated above were the most suitable for our approach to the problem. In the sequel, therefore, it was mainly the excitability at these lower temperatures which was compared with that at 38°.

The material used for the experiments consisted of pieces of gut from rabbits. The results are uniform, apart from the cases where the contrary is reported. In judging the results, changes in the intestinal tone and in the magnitude and rate of the movements as well as the duration of the reaction have hitherto been taken into account, but not such factors as the bearing on them of the concentration of the poison and the latency period from the adding of the drug to the bath fluid until the beginning of the response of the intestine.

## Results.

The rabbit intestine was found to be rather insensitive to the lowering of the temperature, but more sensitive to its rise.

The lowering of the temperature from 38° to 34° as a rule entailed no observable change in the working of the intestine during periods of observation of 10—15 minutes. At temperatures of 32°—30°—28°—26°, varying for different guts, the contraction frequency in particular diminishes, but often, though in a lesser degree, also the amplitude. Thus, whereas at 38° the frequency is about 7—8 contractions per minute, the rate at 30°—28°, as a rule, is about 3.4 per minute (Fig. 1). At temperatures of 24°—22° the intestine is still working, but usually with a markedly diminished magnitude and rate of contraction, (Fig. 2). At 30°—28° the magnitude of contraction is often 20—40

per cent. less than at 38°, sometimes, however, actually greater than at the last-mentioned temperature. At 20°—18° the responses are minimal and irregular, or else the intestine is quite motionless (Fig. 2). As a rule, no change in the tonus could be observed.

The raising of the temperature in the water bath from 38° to 40°—41° as a rule entailed no observable change in the working of the intestine during observation periods of 10—15 minutes. If the temperature is further raised the contraetion rate as a rule increases (from about 8 to 10—12 contractions per minute). At

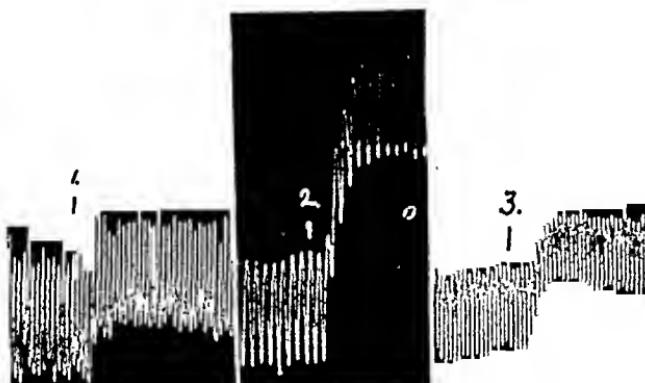


Fig. 1. Isolated rabbits intestine in Tyrode solution. Addition of 0.01 γ/m acetylcholine at 1) 38°, 2) 28° and 3) 38° C.

temperatures between 41° and 46°, varying for different guts the amplitude falls to a minimum with a markedly slowed-down and also often irregular frequency. The range of temperature within which the working of the intestine changes from normal until it completely ceases to function, is as a rule comparatively small, being about 2°—4°. Moreover, the intestine is often so injured by the rises of temperature that repeated tests on the same piece of gut cannot be made. As lowering of the temperature was found to entail more favourable experimental conditions for our approach to the problem, we have hitherto made merely a few preliminary experiments with temperatures over 38°.

The addition of different drugs at varying temperatures to the bath fluid gave the following results.

*Acetylcholine:* On the lowering of the temperature to 32° or 30° in the bath, intensified acetylcholine effects were often obtained. On further lowering to 28°—26° this intensification became considerable, being as a rule of the magnitude and character indicated in Fig. 1, thus with an appreciable increase of the tone.

In cases with less intensification of the effect, it is manifested, in some instances, by greater magnitude of contraction relatively to the increase on an addition of acetylcholine at 38°, in other cases merely by increased tone. The contraction frequency after the addition of acetylcholine remained as a rule unchanged, in experiments both at 38° and at lower temperatures. In isolated cases, however, a slight increase of the frequency after the addition of acetylcholine was observed, when it had previously been markedly low owing to the reduced temperature in the bath.

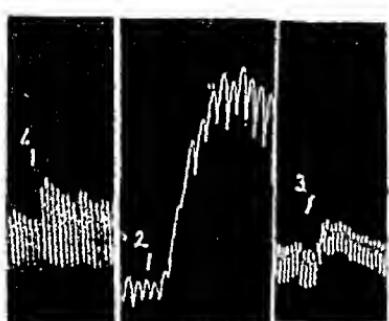


Fig. 2. Isolated rabbits intestine in Tyrode solution. Addition of 0.3 γ/ml pilocarpine at 1) 38°, 2) 24° and 3) 38° C.

Even at very low temperatures (22°—18°), in cases where the working of the gut had actually ceased, a marked contraction was obtained on the addition of acetylcholine in a dosage which at 38° had given a moderate response.

When the temperature was raised above 38°, a moderate reduction of the choline effect ensued at 40°—41°; at 42°—44° this reduction in the effect was pronounced and general, as compared with the effect at 38°. In those cases where

the working of the intestine at these high temperatures was insignificant or nil, the action of acetylcholine doses which at 38° had given a marked effect was completely neutralized. Regularly recurring intensification of the acetylcholine effect at any temperature above 38° has not been observed.

A similar intensification of the intestinal effect at low temperatures as after the addition of acetylcholine was also observed in regard to *pilocarpine* (fig. 2) as well as *doryl* and *mecholyl*. In many cases, however, the intensification was not so marked as for acetylcholine. As regards *physostigmine* (eserine) and *prostigmine*, on the other hand, the said intensification of the effect could not be observed at the lower temperatures; the response of the intestine in many cases began to decrease even at 30°—28° and then diminished according as the temperature was further lowered.

*Epinephrine* showed an intensified effect at 30° as compared with 38°, and in many cases it further increased at about 28°, in order afterwards to fall again. At 22°—24° the action of the

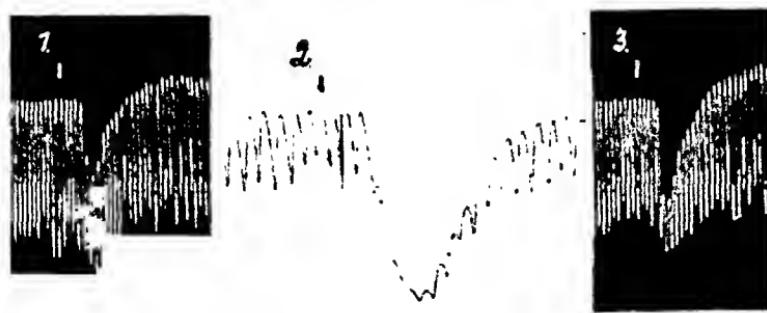


Fig. 3. Isolated rabbits intestine in Tyrode solution. Addition of  $0.01 \gamma/ml$  epinephrine at 1)  $38^\circ$ , 2)  $24^\circ$  and 3)  $38^\circ C$ .

same amount of epinephrine as had shown a marked affect at  $38^\circ$  was greatly weakened or neutralized. When the temperature was raised, an increasing inhibition of the epinephrine effects ensued, as compared with the action of that drug at  $38^\circ$ , beginning as a rule at  $41^\circ$ — $42^\circ$ . At  $44^\circ$  the effect of the epinephrine as a rule was completely neutralized. The above-mentioned intensified action of the epinephrine was manifested especially by diminishing magnitude of the contractions, reduced tonus and prolonged effect, in certain cases also by decrease of the rate (Fig. 3).

A similar intensified reaction at temperatures of about  $28^\circ$  was shown also by *d*- and *l*-corbasil, *dl*-arterenol, *l*-adrianol and *dl*-adrenalon. These intensifications, however, as a rule were not so marked as for epinephrine. *l*-sympatol, generally speaking, did not produce this intensified effect: on two pieces of gut, however, sympatol repeatedly produced the strongest reaction at  $28^\circ$ , as compared with its effect at  $38^\circ$ .

*Ephedrine*, *benzedrine* and *veritol* induced no intensified response at temperatures below  $38^\circ$ . At temperatures of  $30^\circ$ — $26^\circ$ , varying for different guts, the excitability considerably decreased.

As in the case of ephedrine, the maximum reaction of the intestine was at  $38^\circ$  for *atropine*, *ergotamine*, *nicotine*, *substance P* and *histamine* (fig. 4).

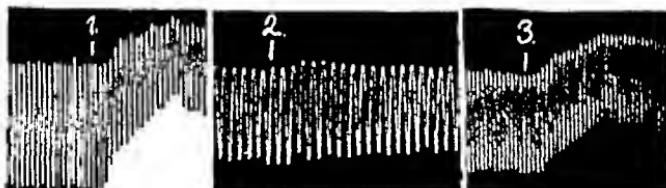


Fig. 4. Isolated rabbits intestine in Tyrode solution. Addition of  $1.2 \gamma/ml$  histamine at 1)  $38^\circ$ , 2)  $28^\circ$  and 3)  $38^\circ C$ .

*Barium chloride.* No certain change in the working of the intestine on the addition of barium chloride at 40° and 42° or 32°, 30° and 28°, as compared with its effect at 38°, could, as a rule, be observed. In certain cases, however, a somewhat weaker action was observed at those temperatures as compared with the effect at 38°. The response of the intestine to barium chloride, generally speaking, diminished rapidly at higher or lower temperatures than those just mentioned.

### Discussion.

The results of this investigation show that the rabbit intestine at different temperatures varies its response to the studied drugs in different ways. The following survey seems to indicate that DALE's theory of neorohumoral transmission can usefully serve as a working hypothesis in the interpretation of the results hitherto obtained.

Acetylcholine and certain *parasympathomimetica* in the proper sense of the term, closely related to it in their mechanism of action as well as *epinephrine* and certain closely related sympathomimetica in contradistinction from the other studied drugs, thus induce a more intense activity at lower temperatures (about 28°) than normal (38°). The group of *parasympathomimetica*, however, are distinguished from the *sympathomimetica* by the fact that the intensified response remains even at such low temperatures (24°—20°) that the movement of the gut are irregular or have completely ceased.

Among the *parasympathomimetica*, pilocarpine as well as doryl, mecholyl chloride and mecholyl bromide, which are chemically related to acetylcholine, likewise induced the said intensified effect at the lower temperatures, though scarcely in the same degree as the acetylcholine. In the case of physostigmine and prostigmine this intensification failed to manifest itself. These results conduce to bear out the view that the three first-mentioned drugs (pilocarpine, doryl and mecholyl) act directly on the effector cells in the same or similar way as acetylcholine, in contradistinction from physostigmine and prostigmine the inhibitors of cholinesterase.

Among the *sympathomimetica* studied, corbasil, arterenol, adrenalon and adrianol showed the same effect as epinephrine, though not always so marked, whereas the so-called pseudo-

sympathomimetica, ephedrine, benzadrine, veritol, and, as a rule, sympathol did not produce this intensification. A parallelism between chemical structure and pharmacodynamic effect can be noted here, in that the intensified effect at the lower temperatures was obtained only as regards sympathomimetica containing a hydroxyl group in the meta-position. The connection between the structure and the action of the sympathomimetica has been previously subjected to numerous investigations. Here it need merely be mentioned that EMILSSON (1942), in investigations regarding the inhibiting action on the isolated small intestine of the rabbit found the same grouping of these sympathomimetica as has just been indicated.

*Atropine*, unlike acetylcholine, does not induce any intensification of the effect on the intestine at about 28°. This observation seems to bear out the view that atropine, in its prevention of the muscarinic effects of acetylcholine and its esters does not directly act on the same structures of the effector cells as acetylcholine.

The experiments with *ergotamine* seem likewise to indicate, that this drug does not attack the same structure of the effector cell as epinephrine.

Barium chloride, which stimulates muscles of all types, regardless of innervation, did not produce any intensified effect at the low temperatures in question, nor did histamine or substance P.

In attempting to explain why the rabbit intestine at varying temperatures changes its response to different drugs in diverse ways, it seems plausible to suppose that different kinds of "receptor substances", or *reactive material of the effector cells*, show dissimilar resistance to low temperatures.

On the other hand, it seems less probable that the cause of the intensified effect at the lower temperatures is to be sought in diminished enzymatic breaking-down of these drugs (eholinesterase, amino-oxidase etc.). The result of the eserin experiment argues against such a supposition.

Preliminary tests with the same method on other organs (the uterus of guinea pigs and rabbits, guinea pig intestines) have not verified the results obtained on rabbits. The effects are also small relatively to those on rabbits, nor can any certain difference in the action of different groups of drugs be shown in regard to these organs. It therefore seems as if, among the organs hitherto tested, only the rabbit intestine were suited for studies of this nature.

### Summary.

The action of a number of drugs on the isolated, surviving small intestine of the rabbit was studied at different temperatures according to the method of MAGNUS.

Acetylcholine and some closely related parasympathomimetica (pilocarpine, doryl, mecholyl) showed at temperatures roundabout 28° a stronger effect on the intestine than at 38°. The intensified action of those drugs was manifested even at such low temperatures (24°—20°) that the intestine worked irregularly or stopped working altogether. On the addition of physostigmine and prostigmine, on the other hand, these intensified effects were not produced.

Epinephrine and certain sympathomimetica closely related to it in structure (corbasil, arterenol, adrianol and adrenalol) showed a maximum effect at temperatures roundabout 28°. This intensified effect, however, was not retained at the lower temperatures. As regards the so-called pseudosympathomimetica (ephedrine, benzedrine and veritol) no increased effect was produced on lowering the temperature below 38°. The intensified effect at the lower temperatures was obtained only as regards sympathomimetica containing a hydroxyl group in the meta-position.

As for atropine, ergotamine, histamine and substance P, no intensified effect was induced.

The dissimilar response of the rabbit intestine to different groups of drugs at varying temperature is presumably due to the apparently dissimilar resistance of different kinds of "receptor substances", or reactive material of the effector cells, to low temperatures.

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## On the Synthesis of Proteins in Rat by Dialyzed Casein Digests.

By

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It has been shown among others by HENRIQUES and HANSEN (1905) that nitrogen equilibrium and thus also protein synthesis are easily maintained in rats by enzymatic hydrolysed casein as the sole nitrogen-containing food. In order to make such casein digests free from undigested proteins and high-molecular peptides, the author (1944, 1945) has described a method for dialyzing the hydrolysates through cellophane membranes. By this procedure a product is obtained which contains 80—85 % free amino acids and 15—20 % low-molecular peptides. It was in order to show that amino acids produced in this manner are able to maintain nitrogen equilibrium and also to enable protein synthesis in rats that this investigation was made.

### Method.

For the experiments adult rats have been used. The animals were kept in cages of the kind described by HENRIQUES and HANSEN (l. c.). To the vessel used for collecting urine from the rat was added 10 ml of a saturated solution of boric acid. Every day at ten o'clock the urine was collected and diluted to 200 ml with distilled water. From this solution 20 ml was taken for nitrogen determination. The faeces were transferred directly to

the combustion vessel. The nitrogen of both urine and faeces was determined according to the method of Kjeldahl as described by PETERS and VAN SLYKE (1932).

As nitrogen-free food, butter was used together with sugar and salts. The proportions of the ingredients are shown by the protocol of each experiment. The butter and sugar (cane-sugar) were ordinary commercial products. As salt mixture, the one described by OSBORNE and MENDEL (1919) was used. The butter was melted and the sugar and salts were added while stirring, which was continued until the butter had solidified. It was found that this composition contained small amounts of nitrogen, the amount of which was determined in each experiment.

In order to obtain solid faeces the rat daily got cellulose in the form of filter-papers cut into small pieces and mixed with the other food. By analysis it was found that the cellulose contained 0.33 mg nitrogen in 1 g. Also in this case the nitrogen was determined according to the method of Kjeldahl.

An amino acid preparation made by the above-mentioned method was used.<sup>1</sup> The nitrogen content of this preparation was 12.7 %. The amino acids were made to a 25 % suspension by adding distilled water. The pH of the suspension was about 7.1. The nitrogen-content of this suspension was estimated by the method of Kjeldahl. To the food was added amount corresponding to the nitrogen which may be found in the tables.

The amount of the butter, sugar and salt mixture per diem must be large enough to ensure the needed calories but not so large that the rat does not eat all its food in 24 hours. This is a rather difficult task, but with some experience one may find the amounts needed for rats of different weights.

Cellulose was given in amounts of 0.5 to 1.0 g. No difference could be observed in the faeces whether 0.5 or 1.0 g was used.

As a rule, the rats got the amino acids in quantities corresponding to from 130 to 182 mg nitrogen, which is a good deal more than the minimal output of nitrogen. This was done to show the nitrogen retention more clearly.

Water was given ad libitum.

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<sup>1</sup> The commercial product, Aminosol, made by this method has been used in this investigation. Aminosol is prepared by Vitrum, Stockholm.

### Experiment I.

An adult rat weighing 115 g was taken directly to the experiment from an ordinary diet. As nitrogen-free food, a mixture of 250 g of butter, 40 g of sugar and 15 g of salts was used. The rat was given amounts of this composition ranging between 4.00 and 6.00 daily. 1 g of this mixture contained 0.893 mg N. 1 g cellulose per diem was also given corresponding to 0.33 mg N. The amounts of nitrogen given as amino acids will be found in the table.

Table I.

Date 1945	Weight of the rat g.	Butter sugar salt mixture g.	mg N in cellulose, butter, sugar, salt	mg N in the aminoacids	mg N total given	mg N in the urine	mg N in the faeces	mg N total excreted	mg N Difference between N given and excreted
5. 7.	115	6.00	5.69	133.6	139.29	49.84	39.81	89.15	+ 50.14
6. 7.	117	6.00	5.69	133.6	139.29	71.40	33.49	104.89	+ 34.40
7. 7.	119	4.00	3.90	80.19	84.09	73.64	29.12	102.76	- 18.67
8. 7.	111	5.00	4.80	106.9	111.70	79.80	50.96	130.76	- 19.06
9. 7.	111	6.00	5.69	133.6	139.29	72.80	23.80	96.10	+ 43.19
10. 7.	110	6.00	5.69	133.6	139.29	57.50	29.12	86.62	+ 52.67
11. 7.	110	6.00	5.69	133.6	139.29	77.08	49.50	126.58	+ 12.71
12. 7.	113	6.00	5.69	133.6	139.29	65.26	42.22	107.48	+ 31.81
13. 7.	113	6.00	5.69	133.6	139.29	66.82	49.50	116.82	+ 22.97
14. 7.	115	6.00	5.69	133.6	139.29	87.09	39.81	126.40	+ 12.89
15. 7.	117	6.00	5.69	133.6	139.29	97.02	30.77	127.79	+ 11.50
16. 7.	118	6.00	5.69	133.6	139.29	77.70	43.68	121.38	+ 17.91
17. 7.	120	6.00	5.69	106.9	112.59	66.82	29.12	95.94	+ 16.65

From table I it may be seen that during the 13 days of the experiment the rat retained 269.11 mg nitrogen, corresponding to 1.7 g of proteins. As the rat gained 5 g in weight it may be assumed that it got enough calories.

### Experiment II.

The rat weighed 140 g. The butter, sugar and salt mixture was made up of 240 g of butter, 80 g of sugar and 15 g of salts. 1 g of this mixture contained 0.812 mg N. As in experiment I, the rat got 1 g of cellulose (0.33 mg N) daily. The amino acids were given as in experiment I. However the rat did not get any amino acids on the first day of the experiment. In table II it is found that in

14 days (from 20.10 to 2.11) the rat retained 467.66 mg N, corresponding to 2.9 g of proteins. In this experiment the rat gained 7 g in weight.

Table II.

Date 1945	Weight of the rat g.	Butter sugar salt mixture g.	mg N in cellulose, butter, sugar, salt	mg N in the aminoacids	mg N total given	mg N in the urine	mg N in the faeces	mg N total excreted	mg N Difference between N given and excreted
19. 10.	140	5.00	4.73	—	4.73	81.2	11.73	92.93	— 88.20
20. 10.	142	5.00	4.73	182.0	186.73	144.2	45.57	189.77	— 3.04
21. 10.	140	5.00	4.73	182.0	186.73	143.2	26.25	169.45	+ 17.28
22. 10.	140	5.00	4.73	182.0	186.73	133.4	27.87	160.77	+ 25.96
23. 10.	140	5.00	4.73	182.0	186.73	126.0	28.42	154.42	+ 32.81
24. 10.	140	5.00	4.73	182.0	186.73	116.5	25.49	141.99	+ 44.74
25. 10.	139	5.00	4.73	182.0	186.73	121.2	26.82	148.02	+ 38.71
26. 10.	140	5.00	4.73	182.0	186.73	130.2	31.01	161.21	+ 25.52
27. 10.	140	5.00	4.73	182.0	186.73	140.4	40.95	181.85	+ 5.88
28. 10.	140	6.00	5.20	182.0	187.20	119.9	27.87	147.77	+ 39.48
29. 10.	145	5.00	4.73	182.0	186.73	105.7	31.81	137.51	+ 49.22
30. 10.	144	6.00	5.20	182.0	187.20	110.2	32.20	142.40	+ 44.80
31. 10.	145	6.00	5.20	182.0	187.20	111.0	31.79	142.79	+ 44.41
1. 11.	145	6.00	5.20	177.2	182.40	118.2	23.88	141.58	+ 40.82
2. 11.	147	6.00	5.20	177.2	182.40	94.6	25.68	120.28	+ 62.12

## Experiment III.

It was in order to see how much nitrogen would be excreted if the animal only received the butter, sugar and salt mixture that this experiment was performed. A rat weighing 125 g was given about the same amounts of calories as in the experiments I

Table III.

Date 1945	Weight of the rat g.	Butter sugar salt mixture g.	mg N in cellulose, butter, sugar, salt	mg N in the urine	mg N in the faeces	mg N total excreted	mg N Difference between N given and excreted
5. 11.	125	5.00	4.23	82.74	38.50	121.24	— 117.01
6. 11.	120	5.00	4.23	58.38	?	?	— ?
7. 11.	115	5.00	4.23	32.90	22.46	55.86	— 51.18
8. 11.	110	5.00	4.23	36.96	22.16	59.12	— 54.89
9. 11.	110	5.00	4.23	35.84	14.98	50.82	— 46.59
10. 11.	110	5.00	4.23	41.58	12.61	54.19	— 49.96
11. 11.	110	5.00	4.23	39.62	13.61	53.28	— 49.00
12. 11.	110	5.00	4.23	35.70	9.57	45.27	— 41.04
13. 11.	108	5.00	4.23	39.62	8.82	47.94	— 43.71

and II. The composition of the butter, sugar and salt mixture was 240 g of butter, 80 g of sugar and 15 g of salts. Of this mixture amounts of 5.0 g were given. 1 g of this compound contained 0.812 mg N. Besides this 0.5 g of cellulose, containing 0.17 mg N was given. This experiment was broken off after 9 days because the rat did not eat its food.

From table III it may be seen that the small amounts of nitrogen in the butter sugar and salt composition and in the cellulose are of no importance with regard to the nitrogen equilibrium and the nitrogen-retaining power of the amino acids in the experiment I and II. The nitrogen quantity given in the butter, sugar and salt mixture and the cellulose was always below 10 % of the excreted amount of nitrogen, and it is thus impossible to get a nitrogen equilibrium with this small amount of nitrogen.

### Summary.

It has been shown that dialyzed enzymic casein hydrolysate could give not only nitrogen equilibrium but also a considerable nitrogen retention in rats.

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## Effect of Acetylcholine and Adenosine Triphosphate on Denervated Muscle.

By

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Received 2 February 1946.

In previous papers (BUCHTHAL et al. 1944 a b c) it could be demonstrated that minute amounts of adenosine triphosphate (ATP) release contraction in frog and mammalian striated muscle. It was suggested that ATP constitutes a further link in the chain of reactions initiated by the injection of acetylcholine into the artery of a muscle. This high energetic phosphate is even effective after complete curarization. Apart from the release of contraction by ATP another property of this substance was disclosed, viz. its sensitizing effect on subsequent applications of acetylcholine. Thus, it seemed likely that in some way or other more intimate interactions take place in the nerve muscle system between acetylcholine and ATP. We thought it of interest to investigate the effect of ATP on denervated muscle, as denervation causes profound changes in the reaction to acetylcholine. It is generally accepted that normal skeletal muscle is relatively insensitive to intra-arterially injected acetylcholine, while denervated muscle after application of minute amounts of this substance exhibits a considerable increase in tension and duration of contraction.

### Method.

The experiments were performed on the anterior tibial muscles of the cat under chloralose or decerebrate as described by BROWN (1938). The substances were applied by close arterial injection into the distal part of the anterior tibial artery, the proximal part being temporarily closed by traction on a ligature, when the substances are applied. The tension developed by tibial muscles on intra-arterial injection of acetylcholine may show rather large individual differences in different cats. It therefore seemed of special interest to record alternately within

short intervals the tension developed by normal and denervated muscles, thus enabling a quantitative comparison under standardized conditions. Therefore, both tibial muscles were mounted in the Brown-Schuster myograph exerting tension on the same isometric lever.

Section of the right sciatic nerve was performed 6—85 days before the experiments in all 25 cats. The completeness of the denervation was controlled by electrical stimulation of the peroneal nerve. The reduction in weight of the denervated muscle amounted to 25—60 per cent as compared with the normal muscle, depending on the time allowed for degeneration.

All substances were applied iso-osmotically by substituting an equal amount of NaCl + water in the Tyrode solution by the staple solution of ATP. The pH of the injected solution was 7.3 and its temperature 37° C. The ATP was applied as sodium salt (for description of preparation and analysis cf. BUCHTHAL et al. 1944 a). The substance was kindly provided by Dr. A. DEUTSCH, research laboratory A. B. Leo, Helsingborg.

## Results.

### *1. Sensitivity to acetylcholine.*

Close arterial injection of minute amounts of acetylcholine into denervated muscle releases a double mechanical response, consisting of a quick initial phase followed by a protracted development of tension. The quick phase is generally absent at the second injection, but may exceptionally persist during the first three applications. This is in agreement with the observations of BROWN (1937). From the 6th day onwards after denervation no systematic correlation exists between duration of tension development and time allowed for degeneration. However, in 3 of 25 experiments with a degeneration period of 46, 47 and 52 days respectively, an exceptional behaviour was observed, the muscle responding to acetylcholine solely by contractions of normal type and duration. In another series of experiments comprising 3 animals with a similar degeneration time of 46, 47 and 49 days resp. the denervated muscle responded with contractions of the protracted type.

The present technique allows a direct comparison of the sensitivity of the normal and denervated muscle to acetylcholine on one and the same animal. The difference in threshold was considerable, the denervated muscle being 20—200 times more sensitive than the normal.

The blocking action of acetylcholine on subsequent injection of this substance is well known from normal muscle. In denervated muscle it is even present when injections are made with intervals of several minutes, denervated muscle becoming in-

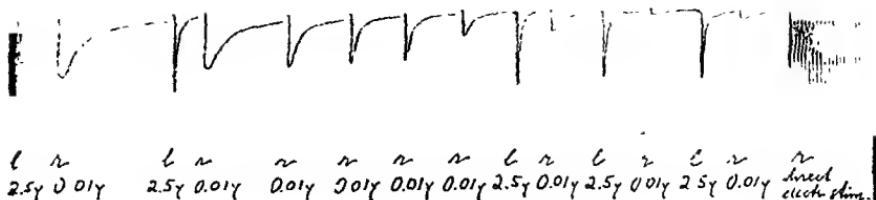


Fig. 1. Cat under chloralose. Mechanical responses from right (r) and left (l) anterior tibial muscles to close arterial injection of acetylcholine. Right muscle denervated four weeks previously. Time marks 1 per second. To the right: direct electrical stimulation of the denervated muscle. Weights: l = 6.4 g; r = 3.0 g.

sensitive to acetylcholine after 10—20 injections. The response to direct electric stimulation is retained (Fig. 1).

## *2. Effect of Na-adenosine triphosphate.*

A solution of ATP injected into the artery in amounts causing submaximal responses evokes contractions of different types in normal and denervated muscles. Compared with the response in normal muscle ATP contractions of denervated muscles are considerably prolonged, the difference in duration being of a similar ratio as that observed with acetylcholine (Fig. 2). As is the case for the latter, duration of contractions released by ATP, from the 6th day onwards, is independent of the time allowed for degeneration. In ATP contractions there is no quick phase even with the first injection. The peak of tension developed during contractions, with the same dose, was generally 30—50 per cent higher in denervated — though atrophied — muscle than in the corresponding normal muscle. A definite small amount of ATP, inactive in the normal, causes a pronounced contraction in the corresponding denervated muscle (Fig. 2). There is, however, no parallelism in the sensitivity of normal and denervated muscle to acetylcholine and ATP. Thus, it was frequently observed that a muscle relatively insensitive to acetylcholine developed strong tension when ATP was injected or vice versa.

In denervated muscle, contrary to findings in normal, previous injection of ATP does not enhance the tension developed by subsequent application of acetylcholine.

In the course of this investigation evidence accumulated that under certain conditions ATP was ineffective in eliciting mechanical responses in denervated muscle. This was always the case when previously to the application of ATP acetylcholine had been injected into the muscle.

The response to acetylcholine and to direct electrical stimulation is, however, retained. For one experiment of this type a supply of lithium ATP was available and it was striking that Li-ATP was active even after previous injection of acetylcholine when the muscle was refractory to Na-ATP.

This is in agreement with our observations on smooth muscle where Li-ATP proved effective in cases where the action of Na-ATP was strongly reduced (BUCHTRAL and KAHILSON 1944).

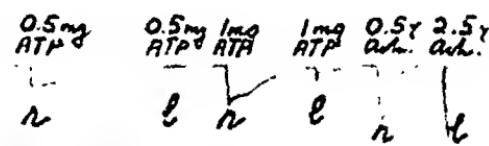


Fig. 2. Cat under chloralose. Mechanical responses from right (r) and left (l) anterior tibial muscles to close arterial injection of acetylcholine and ATP. Right muscle denervated five weeks previously. Time marks 1 every ten seconds. Weights:  $r = 7.0$  g;  $l = 2.8$  g.

### Discussion.

The aim of this investigation was a direct comparison of the reaction of normal and denervated muscle to injections of acetylcholine and ATP. Independent of the time of degeneration both substances release a contraction of long duration in denervated muscle. It is well known that acetylcholine, ineffective in initiating contractions in curarized muscle, evokes a special type of response in denervated muscles. Since curarization, in normal muscle, does not alter the type of contraction, it is obvious that denervation, apart from changes in the peripheral nerves and motor end plates causes changes in the reaction of the muscle substance itself to chemical stimulation. It is on the other hand worth mentioning that the greater sensitivity of denervated muscle to chemical stimuli is abolished by curarine (BROWN 1937) and even after a degeneration period of almost 3 months, the motor end plate still is highly sensitive to acetylcholine.

The depressing effect of acetylcholine on subsequent application of ATP in denervated muscle indicates that acetylcholine interferes with the reaction of the muscle substance. As the response to acetylcholine is retained when the preparation has be-

come refractory to ATP, we are forced to suppose that in denervated muscle acetylcholine prevents the interaction of the *intrarterially* applied ATP with the contractile substance. The mechanism of this inhibition remains obscure. It may, however, be noted in this connection that acetylcholine actually interferes with the enzymatic activity of myosin. Acetylcholine has a considerable inhibitory influence on the adenosinetriphosphatase. Furthermore, previous application of acetylcholine abolishes the changes in birefringence produced by ATP in normal frog muscle fibres. This, too, indicates that acetylcholine, apart from its effect on the motor end plate, in some way reacts on the contractile protein.

### Summary.

Direct comparison of denervated with normal anterior tibial muscles of the cat showed:

1. Sodium adenosine triphosphate initiates contractions in denervated muscle which last considerably longer than in normal muscle.
2. Previous application of acetylcholine to denervated muscle abolishes its sensitivity to ATP, while the reaction of normal muscle to this substance is uninfluenced by previous injection of acetylcholine.
3. In agreement with previous investigations we find denervated muscle considerably more sensitive to acetylcholine (average 40—50 times) than normal muscle; the curare-like action observed on repeated applications of acetylcholine is highly pronounced in denervated muscle. Since the increased sensitivity still is present when degeneration has been allowed to proceed for 75—85 days, and since this effect can be abolished by curarization, it must be concluded that the motor end plate or part of it survives even at this high degree of muscle atrophy.

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## A Modified Preparation of the Universal Buffer Described by Teorell and Stenhagen.

By

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The universal buffer solution for the pH-range 2—12 described by TEORELL and STENHAGEN 1938 has been used in various investigations in this laboratory and has apparently also been found useful in other laboratories. The buffer is intended primarily for biological and surface chemistry work. It is nitrogen-free, the content of cations other than hydrogen ions is constant throughout its range and the variation of buffer capacity and ionic strength fairly moderate. The stock solution for the buffer contains sodium phosphate, citrate and borate, together with an excess of sodium hydroxide. In order to prepare a buffer solution with a desired hydrogen ion concentration, a certain volume of stock solution is taken, the appropriate amount of hydrochloric acid added and the mixture diluted to a standard volume. As originally described, the sodium phosphate and citrate needed for the stock solution are obtained by titrating 100 ml of 1-N sodium hydroxide with a solution of phosphoric and citric acid respectively. This procedure has to be used when suitable buffer salts are not available. When buffer salts according to SÖRENSEN are accessible it is more convenient to use the latter and thus avoid the titration procedure. We have therefore prepared a series of stock solutions in this manner and, as the two modes of preparation may not be exactly equivalent, remeasured the buffer over the entire pH-range.

## Experimental.

*Potentiometric measurements.* The measurements were carried out at a temperature of  $20.0^\circ \pm 0.1^\circ$  (well-stirred water bath) with the use of cells consisting of hydrogen electrodes (palladium black electrolytically deposited on platinum foil, cf. CLARK 1928) and 3.5-N calomel electrodes. A series of 3.5-N calomel electrodes were prepared following the directions given by BJERRUM and UNMACK 1929. Calomel was prepared electrolytically from purified mercury. The calomel electrodes did not differ by more than 0.1 millivolt from each other and they had a potential of 0.3717 volt against the hydrogen electrode in a solution of 0.01-N HCl, 0.09-N NaCl (Veibel's solution) in exact agreement with that found by BJERRUM and UNMACK. The hydrogen for the bubbling electrodes was taken from a commercial cylinder and purified by passing through an electric oven with a filling of copper to remove oxygen. Before entering the electrode vessels the hydrogen was saturated with moisture by passing through a washing bottle filled with conductivity water and immersed in the bath. The potential measurements were carried out using the "Slide wire potentiometer" and Weston standard cell made by the Cambridge Instrument Co. and a Lange's Multiflex galvanometer of high sensitivity. Readings were taken to 0.1 millivolt. For calculation of pH-values, the potential of the 3.5-N calomel half-cell was taken as 0.2522 volt. For a discussion of the standardization of the pH-scale the reader is referred to a paper of MAC INNES, BELCHER and SHEDLOVSKY 1938. The liquid junction potential of the 3.5-N KCl-bridge was neglected.

## Preparation of Buffer Solutions.

*0.1-N Hydrochloric acid.* Constant boiling hydrochloric acid prepared according to HULETT and BONNER 1909 is diluted to 0.1-N. If the distillation has been carried out at 760 mm pressure, 18.019 g of constant boiling acid is diluted to 1,000 ml. The amount to be taken, when the distillation has been performed at other atmospheric pressure may be obtained from tables given in the paper of HULETT and BONNER or VAN SLYKE and PETERS 1932.

*1-N Sodium hydroxide.* A saturated solution of sodium hydroxide (Eka, analytical reagent) is prepared and centrifuged in glass

tubes provided with rubber stoppers until all insoluble carbonate has been collected at the bottom of the tube. 58 ml of the clear supernatant solution is diluted with carbon dioxide-free conductivity water to 1,000 ml. The strength of the solution thus prepared is determined by titration with the 0.1-N hydrochloric acid described above. It is very important that the acid and the base correspond exactly to each other.

*Buffer stock solution.* In a 1,000 ml measuring flask are placed 8.903 g (0.05 mole) of disodium phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 2 \text{ H}_2\text{O}$ , Kahlbaum, "nach Sörensen" or equivalent), 7.00 g (0.0333 mole) of crystallized citric acid ( $\text{C}_6\text{H}_8\text{O}_7$ , 1  $\text{H}_2\text{O}$ , Riedel-de Haen or equivalent), 3.54 g (0.0507 mole) of crystalline boric acid ( $\text{H}_3\text{BO}_3$ , Kahlbaum or equivalent), 243.0 ml of 1-N sodium hydroxide and carbon dioxide-free distilled water (preferably conductivity water from a special still, such as that described by ELLIS and KIEHL 1935) added to make the volume 1,000 ml. The stock solution is kept in a flask of Pyrex of Jena Geräte glass provided with a 20 ml automatic pipette. The solution must be protected from atmospheric carbon dioxide by efficient soda lime tubes.

*Buffer solutions of desired pH.* A buffer solution is prepared as follows: to 20 ml of the stock solution is added the amount of 0.1-N hydrochloric acid found from table I for the pH in question, and carbon dioxide-free distilled water added to make the volume 100 ml. For example, if a buffer solution of pH .760 is wanted, then 30.33 ml of 0.1-N hydrochloric acid is added to 20.00 ml of stock solution and water added to 100 ml.

Table I.

pH → ↓	.00	.10	.20	.30	.40	.50	.60	.70	.80	.90
2		72.10	69.25	66.87	64.90	63.25	61.77	60.48	59.29	58.29
3	57.49	56.76	56.05	55.42	54.83	54.28	53.72	53.17	52.61	52.07
4	51.52	51.00	50.46	49.92	49.40	48.88	48.35	47.81	47.28	46.72
5	46.18	45.64	45.10	44.54	43.99	43.40	42.77	42.15	41.51	40.89
6	40.28	39.66	39.02	38.31	37.54	36.73	36.02	35.36	34.72	34.13
7	33.51	32.97	32.46	31.90	31.36	30.82	30.33	29.88	29.45	29.06
8	28.70	28.44	28.20	27.91	27.56	27.20	26.83	26.44	25.77	25.12
9	24.48	23.82	23.21	22.60	21.95	21.32	20.71	20.18	19.60	19.10
10	18.65	18.24	17.84	17.51	17.20	16.92	16.68	16.35	15.98	15.56
11	15.09	14.59	13.92	13.08	12.09	10.75				

The modified preparation should give a buffer solution of the same molar strength as that given by the original mode of preparation, *i. e.*

$H_3PO_4$	0.0100	constant in the whole range
$H_3BO_3$	0.0114	
$H_3Cl$	0.0067	
NaOH	0.069	
HCl	0.001 (pH 11.50) to 0.072 (pH 2.00)	

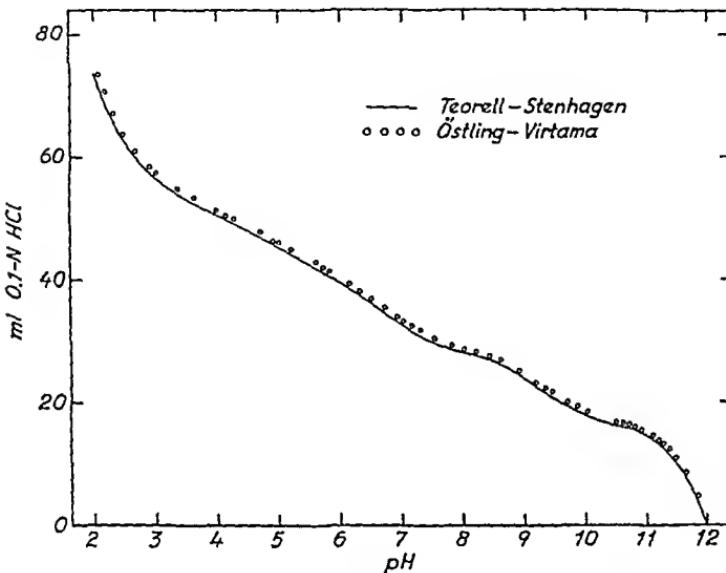


Fig. 1. Variation of pH with addition of 0.1-N HCl to buffer stock solution.

The measurements show small systematic differences, however. In the whole range the modified preparation gives solutions, which are slightly less acidic than those of the original preparation (see fig. 1). Part of the difference (0.032 pH units) is due to the new standardization of pH-scale, the rest is connected with the titration procedure in the original mode of preparation which is likely to give the stock solution a phosphoric acid content slightly higher than that of the modified preparation. The pH-values of the original buffer have been repeatedly checked in the Institute of Physiology and confirmed within the unsystematic experimental error of 0.03–0.06 pH units. The measurements on the modified preparation have been carried out on five different stock solutions, and the agreement between the different series of measurements is within 0.05 pH units.

The variation in ionic strength with pH is shown in fig. 2.

We are indebted to Professor T. TEORELL for the loan of a Multiflex galvanometer and to Docent E. STENHAGEN for advice.

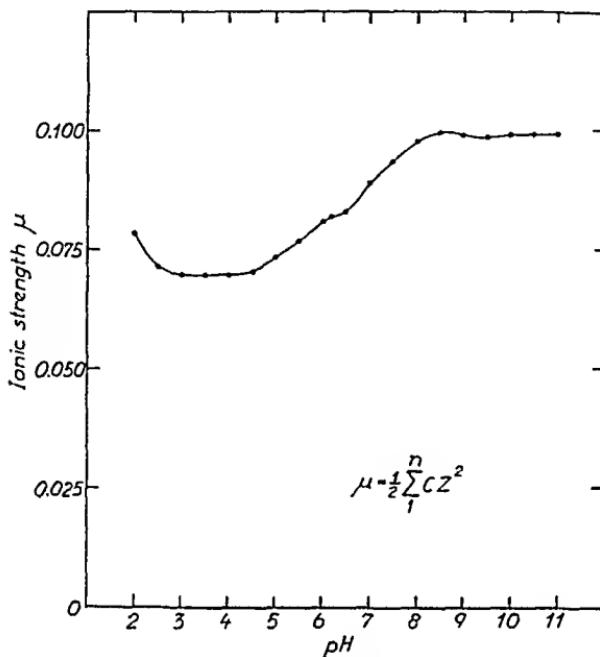


Fig. 2. Variation of ionic strength with pH.

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## A Note on the Biogenesis of Choline and Creatine.

By

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In recent years much work has been expended on the study of the biochemistry of choline, and our knowledge of this remarkable compound is now fairly extensive. One of the most interesting problems encountered in this field of research is that of the biogenesis of choline. The older literature on the problem has been aptly summarised in the book by GUGGENHEIM (1940), and the more recent contributions have been discussed by WERLE (1943) (cf. also STEENSHOLT 1945 a). A historical review of the subject will therefore not be given here.

The pioneer investigations by DU VIGNEAUD, CHANDLER, COHEN and BROWN (1940) which led to the recognition of the essential rôle played by methionine in biological methylation processes, were carried out on intact animals by the method of isotopes. To the present writer it appeared not unimportant to carry out investigations of the same reactions by means of tissue slices or tissue extracts and more direct methods of choline determination, and a report upon some results obtained in this direction was recently given (STEEENSHOLT 1945 a). By *in vitro* experiments it was found that muscle and liver tissue in the rat are able to catalyse the methylation of ethanol amine to choline, the methyl groups being furnished by methionine.

In this work choline was determined by the method of MARENZI and CARDINI (1942). The principle of this method is to precipitate choline as reineckate, and then determine the chromium in the reineckate by a procedure based on the reaction of CAZENEUVE (1900).

Of the other methods of determination of choline we mention here that of BEATTIE (1936) and of ROMAN (1930). BEATTIE's method consists in precipitating choline as reinecke salt and measuring colorimetrically the red colour imparted by this salt to acetone. The method of ROMAN is based on previous work by STANEK, who found that choline can be precipitated by potassium triiodide. A choline periodide is formed, which was recognised as a choline cyanogen-iodide. These findings were confirmed by ROMAN. In his method choline is precipitated in the form mentioned, and the iodide determined by titration with sodium thiosulphate.

The purpose of the present paper is to compare the results on choline synthesis previously obtained by the method of MARENZI and CARDINI to those obtained by the methods of BEATTIE and ROMAN, and thus to corroborate and confirm our previous findings (STEENSHOLT 1945 a).

In all the work referred to above only methionine has been used as methyl donor. However, according to investigations by TOENNIES and KOLB (1939) methionine is easily oxidised to the sulphoxide. This conversion is easily effected by means of hydrogen peroxide, and it is far easier to oxidise methionine than cysteine and cystine [TOENNIES and CALLAN (1939)]. We have therefore to consider the possibility of methionine sulphoxide acting as a methyl donor. In this connection it is very interesting that BENNETT (1941) has shown, by feeding methionine sulphoxide to animals, that it is capable of completely replacing methionine. On the basis of our present knowledge we are entitled to assume that the biological action of methionine is due in large part to its methylating properties, and obviously BENNETT's work leads us to believe that the sulphoxide acts in the same way. The objection might be raised that methionine sulphoxide is reduced to the sulphide in the animal body. However, it is an experimental fact that the sulphoxide is very resistant against reduction by hydrogen iodide. It is therefore quite probable that the sulphoxide exerts its action in the animal organism without previous reduction.

On considering this evidence it may not appear superfluous to investigate by *in vitro* experiments the properties of methionine sulphoxide as a methylating agent. A report on some experiments in this direction will be presented in the present paper.

Methionine sulphoxide is also of interest for another type of methylation processes, which has claimed the attention of bio-

chemists in recent years, namely the biosynthesis of creatine. It is generally believed today that creatine is formed in the animal body mainly by methylation of guanidine acetic acid, the methyl groups being furnished by a suitable donator, probably methionine. The present writer recently had occasion to give a short review of these developments together with some new experimental results on the biosynthesis of creatine [STEEENSHOLT (1945 b)]. From what has been said above it is natural to raise the question whether also methionine sulphoxide is capable of supplying methyl groups for the methylation of guanidine acetic acid. In the present note a report will be given on some results recently obtained on this problem.

Part of our previous work in this field was carried out by a modification of the well known method of FOLIN for the determination of creatine. Due to the defects of this method, in particular its comparative unspecificity, the work was later supplemented by investigations based on a method of creatine determination developed by BENEDICT and BEHRE (1936), Langley and EVANS (1936) and LEHNARTZ (1941). For the convenience of the reader and for the sake of accuracy in the description of experiments, a brief summary of these methods will be given below.

### Experimental Part.

*Biological material.* Rat liver and rat muscles were used throughout. The animals were from 3 to 5 months old, and had been kept on a diet believed to be sufficient in all respects. They were killed by decapitation and the organs to be used removed immediately after death.

*Substrates.* The methionine was a Hoffmann-la Roche product. The ethanol amine was partly a commercial product, carefully purified by repeated distillations, and partly synthesised by the writer according to KNORR (1897). Briefly described this method consists in leading a stream of ethylene oxide through a concentrated aqueous ammonia solution and subsequently fractionating the reaction mixture. Both samples were found to behave in exactly the same way in all the experiments described below.

The methionine sulphoxide was synthesised according to TOENNIES and KOLB (1939) by oxidising methionine by means of hydrogen peroxide in a suitable solvent. The method was found to work very satisfactorily.

Further the guanidine acetic acid used in the present work was synthesised according to NENCKI and SIEBER (1878).

*Determination of choline.* The method of MARENZI and CARDINI is as follows:

The amount of choline to be determined varies between 15 and 100  $\gamma$ . The volume of the sample may range from 1 to 3 ml. The sample is placed in a centrifuge tube with slender end and an equal volume of an aqueous solution of ammonium reineckate is added. The mixture is cooled in ice for 20 minutes, and the precipitate is then spun down in the centrifuge. The supernatant liquid is removed, and the precipitate is washed two or three times with 0.5 ml ice cold alcohol. It is then dissolved in 1 ml acetone and the solution is transferred to an ordinary test tube, the centrifuge tube being carefully washed with some 60 % acetone. We then add: 2 ml of water, 0.2 ml 10 % NaOH and 0.1 ml perhydrol for each 50  $\gamma$  choline in the sample. The tubes are now placed in a boiling water bath for about half an hour. After cooling 2 ml of sulphuric acid are added together with sufficient diphenyl carbazide (in a 0.2 % alcoholic solution) to give a final concentration of 8 %. The mixture is finally transferred to a measuring flask and diluted to a suitable volume, in our work to 25 ml. The colorimetric measurements were carried out with the Pulsfrieh photometer, using filter S 53. The comparison tube contained a blank consisting of 2 ml sulphuric acid and 2 ml diphenyl carbazide solution made up to a final volume of 25 ml.

The method of BEATTIE was applied in the following form:

To 2 ml of the solution to be analysed is added 2 ml of a saturated solution of ammonium reineckate, and the precipitation is completed by cooling in ice for about 20 minutes. The precipitate is separated by centrifugation and washed with 2 ml of ice cold water and twice with 2 ml of absolute alcohol. It is then dissolved in a suitable amount of acetone and the colorimetric measurement carried out in a photoelectric photometer (or according to the instructions given by BEATTIE in her paper).

The method of ROMAN was as follows:

The solution in which choline is to be determined must be neutral or weakly acid. Hence the trichloroacetic acid extracts from our biological experiments were neutralised with the appropriate amount of NaOH. The rest of the determination was as follows:

1 ml of the solution to be analysed was placed in a centrifuge tube with slender end, and 0.3 ml of a precipitation reagent (made by dissolving 3 g of iodine in 100 ml 1-n iodine solution) were added. A brownish precipitate forms, which is separated from the liquid by centrifugation for 10 minutes at 3000 R. P. M. The precipitate is washed 3 times with ice cold water. Then 1 or 2 ml of chloroform is added, and the titration is carried out with n/100 sodium thiosulphate solution. It is a two phase titration, and thorough shaking during the titration is necessary.

*Determination of creatine.* We have throughout determined the amount of total creatinine in the reaction mixtures. For this purpose we have partly used the method of FOLIN in a modification due to LIEB and ZACHERL (1934 a & b):

2 ml of the solution to be analysed were pipetted into a 10 ml measuring flask. 1 ml n-hydrochloric acid was added and the mixture heated

for 20 minutes in an autoclave at 130° C. After cooling 0.4 ml 10% NaOH and 4 ml of a saturated picric acid solution were added, and the mixture left standing for 10 minutes. Colorimetric measurements were then carried out with the Pulfrich photometer using filter S 53.

The technical details of the other method used for determination of creatine will be described below in connection with the report on a typical experiment.

*Synthesis of choline.* In order to save space we shall restrict ourselves to the description of a single experiment chosen at random from the laboratory journal:

The muscles from the hind legs of a rat were carefully minced. 0.3 g of this minced tissue were placed in a small flask A together with 0.05 ml of ethanol amine, 40 mg of methionine and 4 ml of McIlvaine's phosphate-citrate buffer ( $\text{pH} \sim 7.1$ ). Another flask B contained a blank consisting of exactly the same amounts of tissue etc., but no methionine. They were both incubated for 14 hours at 37° C. The reaction mixture was then deproteinated with 16 ml 10% trichloroacetic acid and the precipitate removed by centrifugation. Choline determinations were carried out by the methods described above. To avoid unnecessary tabulation we give only the increase in choline content in flask A compared to that in flask B expressed in per cent. The results were:

MARENZI and CARDINI method .....	20	%
BEATTIE method .....	19.1	%
ROMAN method .....	22	%

(Double analyses were always carried out.)

When methionine was replaced by methionine sulphoxide in the same amount in the above experiment, all other data remaining unchanged (and also the muscle tissues coming from the same animal), the following results were obtained:

MARENZI and CARDINI method .....	10	%
BEATTIE method .....	8	%
ROMAN method .....	9.5	%

A series of experiments was carried out in which the relative amounts of muscle tissue, ethanol amine and methionine or methionine sulphoxide were systematically varied. The results were always qualitatively the same as in the example quoted above. It was also found that the pH dependence of the reaction was the same both for methionine and methionine sulphoxide.

Work on liver tissue gave similar results.

*Synthesis of creatinine.* A typical experiment was as follows:

Flask A contained:

0.2 g minced muscle tissue  
12 mg guanidine acetic acid  
50 mg methionine  
4 ml phosphate buffer (pH 6.8).

Flask B contained exactly the same amounts of the various components, but no methionine. Both flasks were incubated for 14 hours at 38° C. After this period 4 ml 20 % trichloroacetic acid were added and after centrifugation 2 ml of the liquid were removed for analysis according to the method of LIEB and ZACHERL.

In order to apply the method of BENEDICT-BEHRE-LANGLEY-EVANS-LEHNARTZ an experiment identical with the one just described was carried out, with the following modifications. After the incubation period 4 ml 20 % trichloroacetic acid were added together with 1 ml 10 % hydrochloric acid. The mixture was centrifuged after 1 hour, and 5 ml of the supernatant liquid were autoclaved at 130° C for 30 minutes. After autoclaving the liquid was cooled down and after addition of some methyl red indicator it was exactly neutralised with NaOH. By addition of water the volume was increased to 11 ml. 10 ml of a 6% dinitrobenzoate solution were now added together with 10 ml of a 20 % sodium acetate solution and 1 ml of a 2.5 n NaOH solution. After shaking the flasks were left standing for 5 minutes. Their content was then quantitatively transferred to 50 ml measuring flasks which were filled up to the mark with water. The colour was measured in a Pulfrich photometer.

Similar experiments were carried out with methionine replaced by the same amount of methionine sulphoxide.

Expressing the results, for the sake of brevity, as the percentage increase in total creatinine in flask A compared to that in flask B, we found the following results:

	Methionine	Methionine sulphoxide
LIEB and ZACHERL method .....	10 %	5 %
BENEDICT-BEHRE-etc. method .....	10.5 %	5.6 %

As regards the pH dependence of the process the results for methionine sulphoxide were completely analogous to those previously obtained for methionine. Their main feature was a fairly broad optimum somewhat above the neutral point.

Work on liver tissue gave analogous results, but further numerical details are probably of little or no general interest and are therefore omitted.

### Commentary.

The data presented above show that the three methods used for choline determination yield results as regards the methylation of ethanol amine which are in substantial agreement with one another. The conclusions of a previous paper (STEEENSHOLT 1945 a) are therefore further corroborated. The experiments also show that methionine sulphoxide is actually capable of acting as a methyl donator, but that its efficiency in this respect is considerably less than that of methionine.

The writer is glad to express his best thanks to Prof. R. EGE for his support and generous hospitality.

### Summary.

It is found that methionine sulphoxide is capable of acting as methyl donator in the methylation of ethanol amine to choline and of guanidine acetic acid to creatine. The conclusions of this and of a previous paper are corroborated by application of different methods for the determination of choline and creatine.

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## The Splanchnic Efferent Outflow of Impulses in the Light of Ergotamine Action.

By

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In the course of previous research on the efferent impulse traffic in the splanchnic nerve of the cat (GERNANDT, LILJESTRAND and ZOTTERMAN 1946) we tested in a few preparations the effect of ergotamine in doses of 0.05 to 0.1 mg per kg body weight. As ergotamine injected in these amounts produces a definite change in the response to asphyxiation, we considered that it might be worth while to make a study of the action of this drug, as we now had the possibility of adding some new facts concerning the discharge of efferent impulses in the splanchnic nerve which might serve to elucidate the rather puzzling mode of action of ergotamine.

### Methods.

All the experiments have been made upon cats anaesthetized with 0.06 g chloralose per kg body-weight.

The splanchnic nerve was exposed from the back and the efferent discharge in the nerve was recorded according to the method previously described.

The electric stimulation of the peripheral end of the nerve was performed by different means. Mostly we used break-induction shocks given with Baltzar's apparatus in connection with a coreless induction apparatus as described by LUDWIG (1889). With this apparatus the frequency of stimulation was varied from 30 per minute to 32 per second without changing the strength or duration of the separate stimulus. This strength was varied inversely with the total resistance

of the primary circuit. In a few of our earlier experiments we used a thyratron oscillator driven from the AC mains. This stimulator provided condenser discharges of varying frequency and rate of discharge. Finally, we used a battery-driven stimulator by means of which rectangularly shaped shocks of various frequency could be applied to the nerve. The duration of the shock could be varied from 0.2 to 10 msec, and the strength of the current applied to the nerve was directly controlled by a milliammeter.<sup>1</sup>

The arterial blood pressure was recorded with a Hg-manometer from the femoral or the left carotid artery. Injections were made through the femoral vein. The ergotamine substance used throughout our experiments was the tartrate of ergotamine, Gynergen, manufactured by Sandoz A.G. and the adrenaline used has been the Exadrin produced by AB Astra.

## Results.

### The Effect of Ergotamine on the Efferent Discharge of Impulses in the Splanchnic Nerve.

*Asphyxiation.* As has been previously described (GERNANDT, LILJESTRAND and ZOTTERMAN 1946), asphyxia causes a gradual increase in the efferent discharge of action potentials in the splanchnic nerve. This discharge increases in jerky steps and generally reaches a maximum after about 45 to 60 seconds. After an intravenous injection of ergotamine in doses of 0.05 to 0.1 mg per kg body weight the electric response of the nerve often started earlier, and generally reached a higher maximum within 45 seconds. The general appearance of the electric activity is otherwise very much the same in both cases (fig. 1). It is, however, of great interest to relate this activity to the changes in the arterial blood pressure appearing under these conditions. Whereas normally, asphyxia is followed by a pronounced rise in blood pressure (see fig. 2), there is a fall of blood pressure after administration of ergotamine. Concomitantly with the more rapid increase of efferent impulses in the splanchnic nerve, the blood pressure fell after these relatively moderate doses of ergotamine. This reversal of the effect upon the blood pressure after a dose of 0.05 mg per kg body weight generally vanished after about 45 minutes, but could be reestablished after a repeated dose. For the further analysis of the effect of asphyxia we tested the effect of an excess of carbon dioxide as well as of mixtures low in oxygen

<sup>1</sup> We are indebted to T. Helme, M. A., who has constructed this device, for his kindness in placing it at our disposal.

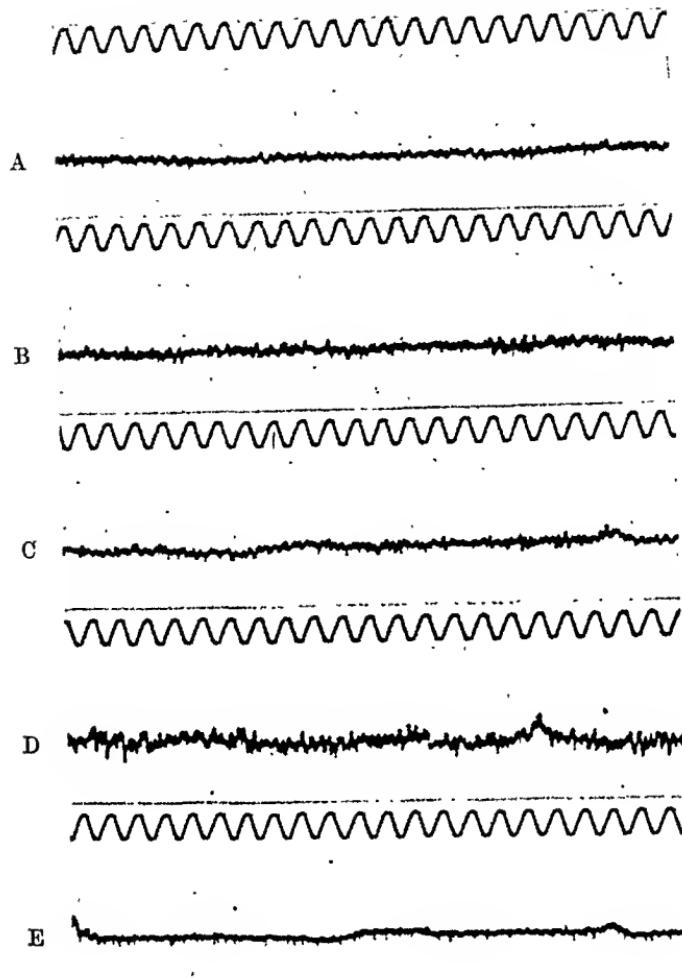


Fig. 1. Cat 2.8 kg. Action potentials from central end of the splanchnic nerve. A. Control, BP 140 mm Hg. B. Art. respiration stopped for 45 seconds, BP 200 mm Hg. C. Control after 0.05 mg ergotamine per kg, BP 150 mm Hg. D. Ditto; art. respiration stopped for 45 seconds. BP now sunk to 110 mm Hg. E. Art. respiration again for 20 seconds, BP rose to 130 mm Hg. The small, very quickly conducted spikes derive from afferent fibres firing from the peripheral cut end of the nerve. Time 50 cycles per second.

on the cat, the blood pressure being recorded from the femoral artery, and the splanchnic innervation left intact. Ten per cent carbon dioxide in air produced a very marked fall in the blood pressure (fig. 3).

Gas mixtures containing less than 8 % oxygen in nitrogen also produced a fall in pressure, but the action was not so pronounced as that of carbon dioxide. Thus it seems as if the depressing effect



Fig. 2. Cat 2.8 kg. Chloralose anaesthesia. This figure is to be read with the electro-neurograms of fig. 1. *A.* The first arrow marks the exposure of the record in fig. 1 *A*. The second arrow marks the stopping of the artificial respiration, the third arrow corresponds to fig. 1 *B*. and the fourth arrow marks the recommencement of art. respiration. *B.* The same animal after 0.05 mg ergotamine per kg body-weight intravenously. The first arrow refers to fig 1 *C*. The second arrow marks the stopping of art. respiration; the third arrow refers to fig 1 *D*; the fourth arrow marks the recommencement of art. respiration; the fifth arrow refers to fig. 1 *E*.

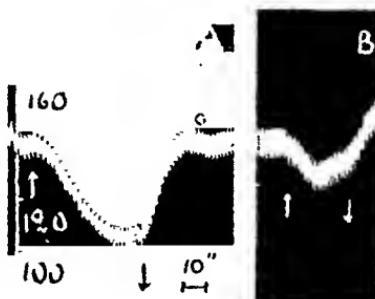


Fig. 3. Cat 2.6 kg. After intravenous injection of 0.05 mg ergotamine per kg body weight. *A.* Between the arrows artificial ventilation with 10.8 %  $\text{CO}_2$  in air. *B.* Between arrows art. respiration with 7.3 %  $\text{O}_2$  in  $\text{N}_2$ .

of asphyxiation on the blood pressure after ergotamine in these doses is due principally to the accumulation of carbon dioxide. This effect was not influenced by denervating the carotid sinus or by severing the vagi in the neck.

#### The Effect of Electric Stimulation of the Splanchnic Nerve.

When the intact nerve is stimulated the strength of stimulus required to elicit a reflex response from the abdominal muscles is far below the strength necessary to induce a rise in blood

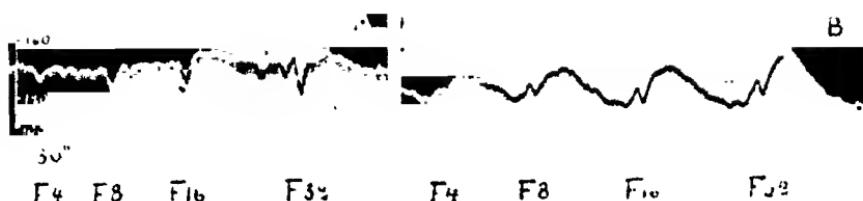


Fig. 4. Cat 2.7 kg. Electric stimulation with break shocks of constant strength but varying frequency to the peripheral end of the splanchnic nerve. Between A and B the XIIth and XIIIth spinal roots were severed. The numbers below the signal give the frequency of shocks per second.

pressure from the peripheral end of the splanchnic nerve. When using rectangular currents it was found that the abdominal reflexes were elicited by currents of low strength and short duration, while a rise in blood pressure could not be elicited from the peripheral end unless the stimuli were of long duration and at least three times that strength.

This result was of course to be expected. The afferent fibres concerned in the abdominal muscle reflex undoubtedly consist of class A fibres, while the constrictor fibres as well as the secretory fibres running to the adrenal glands belong to class B, according to the nomenclature of ERLANGER and GÄSSER.

When the splanchnic nerve was severed above the XIIth thoracic root and stimulated, we found that low frequency stimulation caused a slight drop in the arterial blood pressure. At higher frequencies, above 8 per second, the effect is less, and as the frequency of stimulation increases, the fall is followed by a slight rise in pressure. In these cases, however, there are one or two afferent connections with the spinal cord, via the XIIth or XIIIth spinal roots. When these connections were cut, the same stimulation which previously produced a fall in blood pressure now elicited a rise (Fig. 4). The fall in blood pressure was thus obviously due to the stimulation of afferent fibres running into the XIIth and possibly also the XIIIth thoracic roots, which fibres caused a depressory reflex action in conformity with BRADFORD (1889).

After ergotamine the rise of blood pressure was definitely delayed. After small doses the latency was prolonged for a few seconds, but after a large dose of 0.5—1 mg per kg body weight this retardation was very much pronounced, the latency being prolonged from normally 1 sec. to 25 seconds when 32 induction

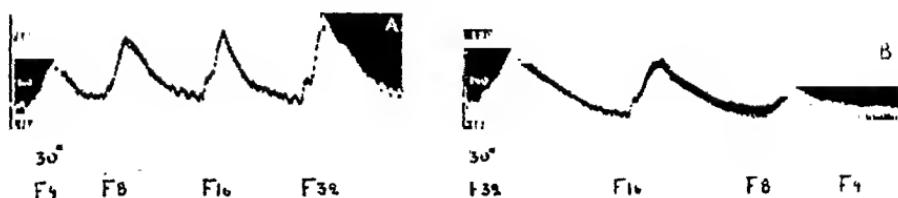


Fig. 5. Cat 3.2 kg. Stimulation as in previous figure. A. before, B. after 1 mg ergotamine per kg intravenously. Note the increase in latency and the retardation of the response after ergotamine.

shocks per second were applied. The pressure rose much more slowly and the duration of the rise was very much prolonged (fig. 5 A and B).

#### The Response to Adrenaline.

After moderate doses of ergotamine, 0.05—0.1 mg per kg body weight, small amounts of adrenaline in doses of 0.25—1  $\mu$  g injected intravenously produced a more accentuated rise in the arterial pressure in accordance with the observation made by EULER and SCHMITERLÖW (1944). The return to the original pressure level also appeared to be somewhat slower. The rise in pressure was accompanied by a diminution of the efferent discharge of the splanchnic nerve (see fig. 1 C). After larger doses of ergotamine, 0.5—1 mg per kg, the effect of small doses of adrenaline was considerably delayed, and the rate of rise much reduced (fig. 6).



Fig. 6. Cat 3.6 kg. A. Control. The effect of 2  $\mu$ g adrenaline. B. After 1 mg ergotamine per kg intravenously. Note the delay in response to the intravenous injection of adrenaline as well as to the stimulation of the peripheral splanchnic nerve.

A reversal of the adrenaline effect we have seen in cats with intact brain and under chloralose anaesthesia only after larger

doses than 2 mg per kg body weight. Even in these conditions the phenomenon is by no means of constant occurrence. Generally, the reversed effect is observed when the level of the blood pressure is high. When the level is below 90 mm Hg we have not seen any reversal, in conformity with CANNON and LYMAN (1913). After pithing the cat the reversal seemed to occur more constantly and seemed to be less dependent upon the level of the blood pressure. In the pithed cat, the reversal was generally seen after 1 mg ergotamine per kg (Fig. 7). With this dose of ergotamine we never obtained any reversal in a cat with intact brain. After larger doses of ergotamine there was generally a very marked increase in the vagal tonus and we therefore often severed the vagi. This procedure did not lead to any change in the response to adrenaline.

There is no reason to expect that adrenaline should exert any direct action upon the vasoconstrictor centres. We also found that the discharge of impulses in the splanchnic nerve changed only in response to the change of blood pressure. Thus when adrenaline caused a rise in blood pressure the discharge was diminished, while the reversed effect of adrenaline after ergotamine led to an increase in this discharge. These changes were very closely related to the change in pressure and not to the amount of adrenaline given. When for example after a large dose of ergotamine (1 mg per kg) there was a very slight rise in blood pressure in response to 0.1 mg adrenaline, the splanchnic discharge was very faintly reduced. Before ergotamine, when the same dose of adrenaline elicited a very high blood pressure, the discharge was entirely wiped out.

After decapitation, when the spinal cord was transsected at C II, there was sometimes a very pronounced increase of the splanchnic discharge (see fig. 8 C). This increased discharge, which had the usual appearance, was always accompanied by a very marked rise in blood pressure to 200 mm Hg. During 10—20 minutes this discharge gradually diminished, and there

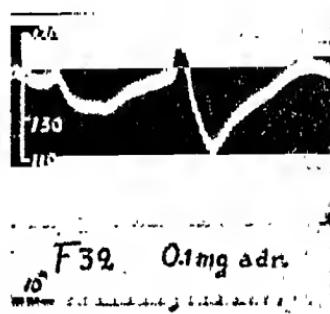


Fig. 7. Pithed cat. 1 mg ergotamine per kg body weight intravenously. The first signal marking refers to faradic stimulation of the peripheral end of the splanchnic nerve with 32 shocks per second for 30 seconds. Thereafter intravenous injection of 0.1 mg adrenaline. Note the *prompt* start of the reversal.

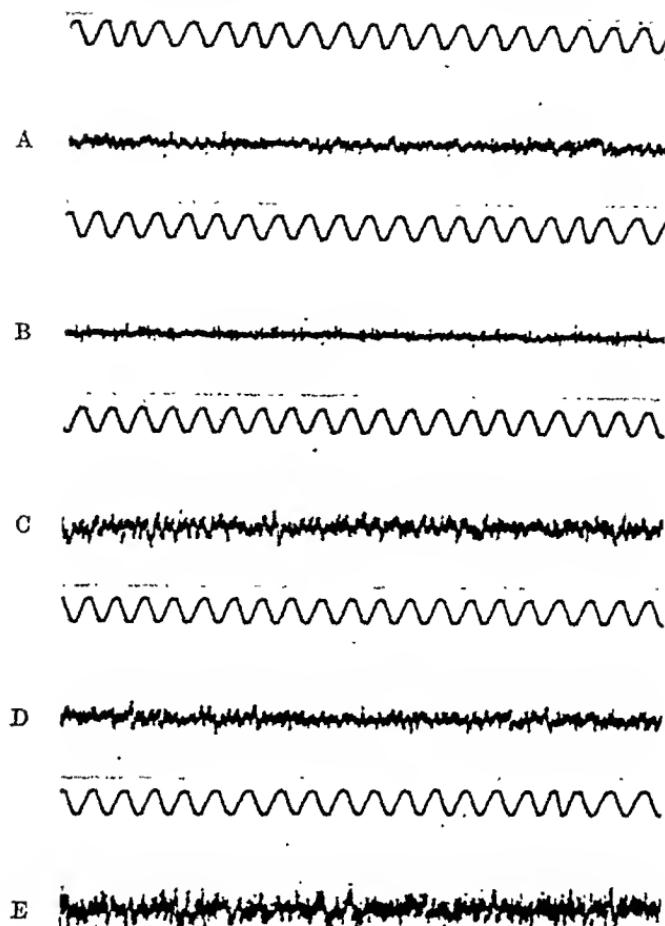


Fig. 8. Cat 2.4 kg. Action potentials from central end of the splanchnic nerve. Distance between off-leads 2 mm. *A*. Control. BP 130 mm Hg. *B*. After 5 µg adrenaline, BP 190 mm Hg. The small diphasic and very rapidly conducted spike potentials seen are injury potentials from afferent fibres. *C*. Immediately after decapitation, BP 180 mm Hg. *D*. 20 minutes later BP 60 mm Hg. *E*. Artificial respiration stopped for 45 seconds. BP rose from 60 to 80 mm Hg. Time 50 cycles per second.

was a parallel fall in blood pressure until the pressure as well as the discharge was stabilized at a lower level (see fig. 8 D). This phenomenon we are inclined to ascribe to a traumatic effect which is gradually diminished.

In the spinal animal asphyxiation elicited a very marked increase in the discharge, and this increase was quite independent of whether the blood pressure rose or fell, the latter being the

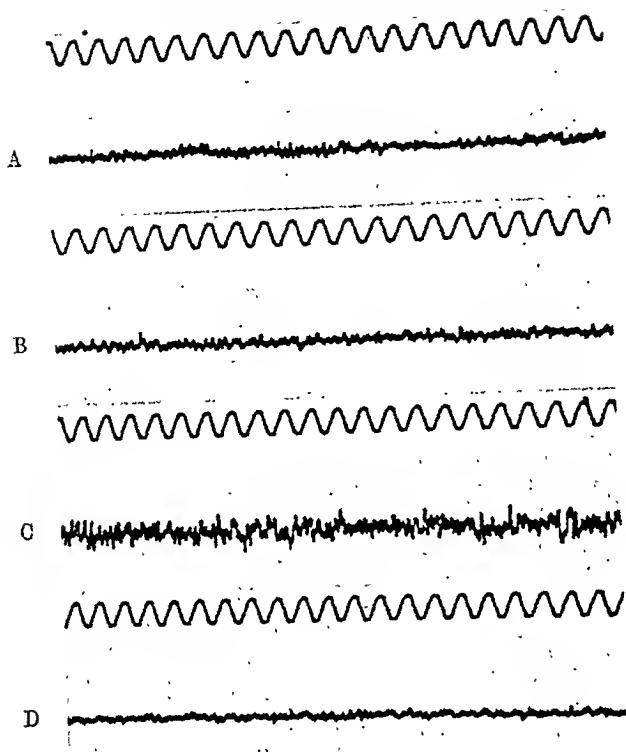


Fig. 9. Action potentials from the central end of the splanchnic nerve of the cat. Same animal as in fig. 8. *A*. After 1 mg ergotamine per kg, BP 90 mm Hg. *B*. After decapitation, BP 110. *C*. Art. respiration stopped for 45 seconds. BP fell to 80 mm Hg. *D*. Art. respiration again for 30 seconds, BP rose to 100 mm Hg.

case after ergotamine in doses of 0.05—2 mg per kg body weight (Fig. 9).

In the spinal cat we could thus not observe that ergotamine produced any other change in the behaviour of the pre-ganglionic response to asphyxia than that the discharge was somewhat increased, which we presume to be due to the fall in pressure.

The splanchnic outflow of efferent action potentials was once observed 10 minutes after the heart had stopped (see fig. 10 A). This activity was found to originate within the spinal cord, as it was completely abolished by cutting the spinal roots (fig. 10 B).

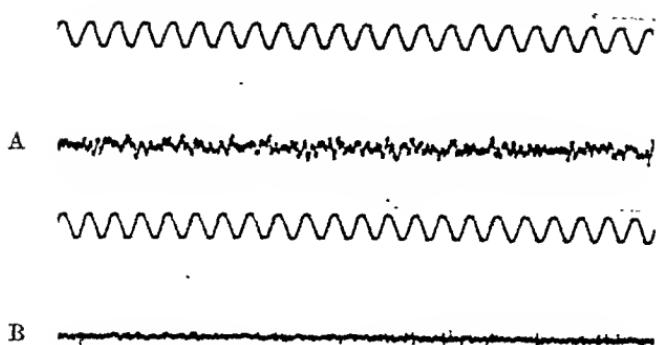


Fig. 10. Action potentials from the central end of the cat's splanchnic nerve. Upper record shows the efferent outflow of impulses 10 minutes after the death of the animal. Lower record immediately after severing the nerve centrally to the off-leads. Distance between electrodes 2 mm. The rapid small spikes seen in the lower curve are antidromic spike potentials from afferent fibres firing from the freshly cut part of the nerve centrally to the electrodes.

### Discussion.

The increased rapidity in the response of the vasomotor centres to asphyxia as observed after even moderate doses of ergotamine is a remarkable fact when correlated with the fact that the action of the increased B-fibre activity in the splanchnic nerve now coincides with a fall in blood pressure. This fall in blood pressure has been clearly proved to be due principally to the accumulation of carbon dioxide, but this fact alone cannot explain the reversed action upon the blood pressure.

According to ROTHLIN (1923) the depressor reflexes are diminished after moderate doses of ergotamine, and v. EULER and SCHMITERLÖW (1944) have produced evidence for the view that this action of ergotamine consists in a centrally located inhibition of the action of the baroceptive impulses upon the vasomotor centre. Thus the readier response of the splanchnic efferent fibres after ergotamine would depend upon the elimination of the continuous inflow of these inhibitory impulses from the aortic and carotid sinus regions. But in order to understand the fact that the augmented fibre activity in the nerve after ergotamine produces a fall in blood pressure, we have to discuss the following possible explanations.

- 1) The impulses observed derive both from vasoconstrictor and vasodilator fibres, but the action of the latter predominates, as the former are inhibited peripherally by ergotamine.

2) The vasoeconstrictor fibre impulses as well as the inereted adrenaline elicit a reversed action directly upon the blood vessels after ergotamine.

The supposition of the existence of specific vasodilator fibres in the splanchnic nerve is based upon the old observations made by BRADFORD (1889) and JOHANSSON (1890), who found that very slow frequency stimulation (30—60 stimuli per minute) of the peripheral end of the splanchnic nerve of the dog often caused a small fall in the arterial blood pressure, while more frequent stimuli raised the pressure. In the cat, however, we have not been able to produce a fall in pressure by low frequeney stimulation of the peripheral end of the splanchnic nerve provided all the afferent conneetions of this nerve with the spinal cord were severed. A fall in blood pressure produced by eleetric stimulation of the peripheral end of the splanchnic nerve we have observed only after large doses of ergotamine (1 mg per kg body weight), and this only after the eat had been pithed. It was only under these conditions that we found a reversed effect upon the blood pressure of intravenous adrenaline injections. After 2 mg per kg body weight the adrenaline reversal was generally observed in eats with the brain intact. In the pithed eat the fall in pressure starts as promptly in response to faradic stimulation as does the rise under normal conditions. In the latter ease the immediate rise is apparently caused by the direct vasoconstrictor response, which is followed by a secondary rise caused by the adrenaline liberated from the adrenal gland. The prompt fall in the blood pressure produced by stimulation of the splanchnic nerve in the pithed cat under large doses of ergotamine occurs with as short a latency as does normally the vasoconstrictor response. Thus it seems as if the first part of the fall were due to a direct action upon the abdominal vessels, in conformity with DALE (1913). DALE interpreted this reversed action in the pithed eat as due to the activity of specific vasodilators which are stimulated concomittantly with the vasoconstrictors, the latter being blocked peripherally by the ergotamine. This fall cannot be produced by using weaker stimuli than those which normally produce the rise, and our experiments with stimuli of various duration and frequeney have not yielded any evidence for the view that constrictor and dilator fibres are different in respect of their reaction to electrical stimulation.

If the constrictor fibres are blocked peripherally by ergota-

mine, the action of the mixed volleys of constrictor and dilator impulses must lead to a fall in blood pressure. With small doses of ergotamine there is a retardation of the rise in blood pressure in response to faradic stimulation of the splanchnic nerve, and this retardation becomes more pronounced as the amount of ergotamine is increased. The important point to remember, however, is that the effect of stimulation as well as the effect of intravenous injection of adrenaline was not reversed until the cat was pithed, or the amount of ergotamine administered was raised to about 2 mg per kg body weight.

Let us now consider the second alternative. We have already mentioned that when there is a reversed reaction to stimulation of the splanchnic nerve after ergotamine it starts very promptly. The same holds true for the reaction to intravenous injections of adrenaline. If we accepted the view of specific dilator fibres we were thus compelled to assume that these fibres were adrenergic in nature. BÜLBRING and BURN (1935) have shown that the leg muscles of the cat are supplied only with adrenergic vasodilators. We should thus accept DALE's original view that ergotamine inhibits the sympathetic motor action but rather exaggerates the sympathetic inhibitory actions, and thus reveals the vasodilator fibre activity. The abolishment of the motor action is proved to be peripheral, but the inhibitory action — in this case the vasodilator action — might be elicited purely peripherally, *i. e.* by the transmitter substance liberated and by the adrenaline secreted from the adrenals. Accepting the theory of sympathin E and I advanced by CANNON and ROSENBLUETH, the increasing massive outflow of impulses in the sympathetic efferent fibres following upon asphyxia must be assumed to produce a peripheral liberation of sympathin E and I as well as an incration of adrenaline from the adrenal glands. Normally, this leads to a vasoconstriction and rise in blood pressure. After ergotamine, however, we may assume that the stimulating action of sympathin E as well as the motor action of adrenaline is reduced or abolished, while the inhibitory action of sympathin I and adrenaline is exaggerated. The increased outflow of efferent impulses will thus produce a reversed effect. The action potentials recorded from the splanchnic nerve must thus be looked upon as being built up by axon potentials deriving from efferent fibres of different functions: a) vaso-constrictor fibres, b) adreno-secretory fibres, c) vaso-dilator fibres (?), d) inhibitory fibres to the intestines. In the peripheral

endings of the first group sympathin E is probably liberated. The adreno-seeretary fibres are obviously cholinergie. The presumed vasodilator fibres and the inhibitory visceral fibres produce sympathin I. Now the sympathin I liberated is normally over-powered in its action upon the blood pressure by the combined action of sympathin E and adrenaline, but after ergotamine the sympathin I activity will be revealed.

These assumptions seem to agree very well with the experience from our experiments in which we directly stimulated the peripheral end of the splanchnic nerve. When applied with adequate strength and frequency the faradic stimulation normally produced a rapid rise in pressure. After ergotamine the rise in blood pressure is delayed and the rise is retarded. This delay in the onset of the rise in pressure is gradually prolonged with increasing doses of ergotamine, until after 1—2 mg per kg the stimulation at once causes a fall in pressure. The responses to adrenaline under these conditions show very much the same course. First there is an exaggerated response due to the inhibition of the depressor reflexes. As the ergotamine doses are increased there is an increasing delay in the response until a point is reached where adrenaline causes the well-known reversal. So far everything seems to fit the scheme.

We have, however, to consider the fact that asphyxia caused a reversed effect upon the blood pressure even after relatively moderate doses of ergotamin. This reversed effect of asphyxia never failed after a dose of 0.05 mg per kg. The fall in pressure is with this dose often not immediate, but is preceded by a small rise. With increased doses of ergotamine the preliminary rise is reduced, however, and finally there is only a direct fall in pressure.

How are we to reconcile this behaviour in response to asphyxia with our above-mentioned view on the ergotamine action? After 0.05 mg or ergotamine per kg the rise in pressure in response to a small amount of adrenaline is rather exaggerated, but the effect of asphyxia is reversed. This does not seem to fit in with the view referred to. We must, however, bear in mind that it is not necessary, or rather it is not correct, to assume that the effect of intravenous injection of adrenaline would have the same action upon the blood pressure as has the increased outflow of efferent impulses in the splanchnic nerve observed during asphyxiation. The motor effect of this discharge can be much more liable to the peripherally depressing action of ergotamin than is the

injected adrenaline. We have further to consider that during asphyxia the accumulating metabolites exert a peripheral vasodilatory action of increasing strength, which normally is overwhelmed by the constrictor and adrenaline outflow. When ergotamine has weakened the motor effect this dilating effect is added to that of sympathin I. This addition brings the reaction upon the blood pressure to a reversal. It is also clear that the direct stimulation of the splanchnic nerve will produce much more massive volleys of impulses than any reflex activity could ever produce. Thus the constrictor effect will win until at last the motor action, or let us say the sympathin E action, is depressed by large doses of ergotamine. The same scheme can be applied to adrenaline. The total abolishment of its motor action demands large amounts of ergotamine.

The question as to whether the splanchnic nerve of the cat contains special sympathetic dilatory fibres to the abdominal blood vessels could not be settled in these experiments, as the electric response recorded did not permit any detailed analysis of its fibre contribution.

The dilator fibres, if they really exist, seem, however, to display the same properties, viz. electric stimulation, as do the constrictor fibres. Thus the possibility of distinguishing the action potentials of the constrictor fibres and the presumed dilator by their shape seems rather remote.

The strongest direct evidence for the existence of specific dilator fibres in the cat's splanchnic nerve hitherto advanced is DALE's demonstration in 1913 that faradic stimulation of the peripheral end after adequate doses of ergotamine caused a slight fall in blood pressure in the pithed cat after removal of the adrenal glands. We have, however, to remember that the faradic stimulation which normally excites the constrictor and the adreno-secretory fibres no doubt affects most of all the other fibres, afferent fibres and inhibitory fibres to the intestinal smooth muscles. The antidromic afferent impulses might thus produce a dilatory action to which the effect of the sympathin I producing inhibitory fibres might add. This would agree very well with the assumption that adrenaline elicits motor as well as inhibitory actions, while the substance produced by constrictor impulses is purely motor-active. It is only the motor activity which is abolished by ergotamine. Thus adrenaline will cause a reversal which sympathin E would not produce. The

effect of sympathin E can thus be expected to be gradually depressed with the amount of ergotamine given and sympathin E will never cause a reversal.

We thus conclude that it seems rather likely that the increased discharge of action potentials following upon asphyxiation before as well as after ergotamine derives from the same fibres. They would thus derive from vasodeconstrictor fibres, adreno-seeretary fibres and inhibitory fibres to the intestines. The reserved reaction after ergotamine to this increased discharge thus seems to be entirely peripherally localized. The simplest explanation would therefore be to assume that the sympathetic nerve activity is exerted via a transmitter which has all the properties of adrenaline. To this the adrenaline liberated from the adrenals will add its action.

After ergotamine in sufficient doses the efferent outflow of sympathetic impulses elicited by asphyxiation or by direct faradic stimulation of the splanchnic nerve will thus produce the same reversed effect upon the blood pressure as does adrenaline injected intravenously.

It must, however, be admitted that all the observations here related can be understood equally well by assuming that the sympathetic motor action is caused by CANNON's and ROSENBLUETH's sympathin E and the inhibitory action by sympathin I, and that adrenaline can give rise to both actions. The efferent outflow which normally raises the blood pressure via sympathin E and the adrenaline secreted from the adrenals will not be able to exert this effect after ergotamine which depresses or blocks the action of sympathin E as well as the motor action of the adrenaline liberated from the adrenal glands. This adrenaline will now exert its sympathin I activity. As DALE (1913) has demonstrated, however, faradic stimulation of the peripheral end of the splanchnic nerve causes a slight fall in blood pressure after removal of the adrenal glands. As discussed above, this effect may be due to a stimulation of afferent fibres and inhibitory fibres running to the intestines. The former fibre would cause a peripheral liberation of acetylcholine and the latter of sympathin I, which might affect the blood vessels as well as the intestinal muscular coat.

When we assume that the sympathetic nerve activity peripherally is mediated by a transmitter sympathin E or sympathin I, it seems that we must reckon with the possibility of specific

sympathetic vasodilator fibres in the splanchnic nerve or assume that the inhibitory fibres to the intestines exert a dilatory effect in order to understand the reversed effect after ergotamine. If we assume that the constrictor fibre activity is mediated by adrenaline, the assumption of specific dilatatory fibres in the splanchnic nerve is unnecessary.

Thus the problem as we see it is not whether there exist dilator fibres in the splanchnic nerve which may be excited electrically, but whether there are specific dilator fibres with no other function.

In such a case one would expect that a rapid rise of the blood pressure as produced by an adequate dose of adrenaline in the normal cat would via the depressor reflexes lead to an outflow of dilator impulses in the splanchnic nerve. In conformity with ADRIAN, BRONK and PHILLIPS 1932 we found that the effect was a complete inhibition of the ordinary outflow and nothing else.

Thus, our study of the splanchnic impulses has not afforded any direct evidence for the existence of specific vasodilator fibres in the splanchnic nerve of the cat, and as the outcome of the above discussion we must conclude that their existence seems to us to be still more uncertain than before.

### Summary.

For an analysis of the action of ergotamine upon the arterial blood pressure of the cat we have recorded the action potentials from efferent fibres of the splanchnic nerve.

When ergotamine is given in a moderate dose of 0.05 mg per kg body weight, asphyxiation or the inhalation of air rich in carbon dioxide produces a fall instead of the usual rise in blood pressure. The electric response, however, differs from that to ergotamine only in that the response now starts earlier and is more accentuated.

After a pithing of the brain and the medulla oblongata the efferent outflow in the splanchnic nerve reacts to asphyxiation and carbon dioxide in a way very similar to the reaction in the intact cat, but the response is more rapid and more pronounced than in the intact animal. Ergotamine does not produce any change whatever in the splanchnic efferent outflow in the spinal cat.

This shows that inhibitory influences upon the spinal vaso-motor centres are exerted from higher centres, which influence

is abolished by ergotamine even in moderate doses, as has been shown by ROTHLIN, WRIGHT and EULER and SCHMITERLÖW.

When giving increasing doses of ergotamine it was found that the effect of faradic stimulation of the peripheral end of the splanchnic nerve, which normally causes a rapid rise in the blood pressure, became more and more delayed and depressed, until after large doses the effect was reversed. At this stage the adrenaline effect was also reversed.

These observations have been discussed in the light of the prevailing theories on the nature of peripheral sympathetic effect.

It was found that the simplest explanation would be to assume that the sympathetic nerve activity leads to a peripheral liberation of adrenaline. The phenomena observed under ergotamine may, however, be equally well understood by assuming that the constrictor excitation is mediated by sympathin E. In such case we may assume that the vasodilation produced by direct stimulation of the splanchnic nerve is due either to the action of antidromic afferent impulses or to the liberation of sympathin I in fibres running to the intestinal walls, or to a combination of both these influences. Thus even in this scheme the supposition of specific sympathetic vaso-dilator fibres in the splanchnic nerve of the cat does not seem to be necessary.

The recording of the action potentials from the splanchnic nerve has not yielded any proof of any activity of vasodilatory fibres either before or after ergotamine.

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## On the Effect of Some Pigments and Redox Systems on the Respiration of Animal Tissue.

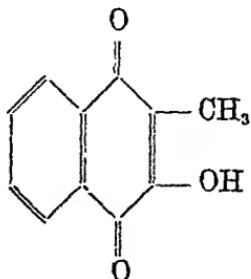
By

GUNNAR STEENSHOLM.

Received 23 February 1946.

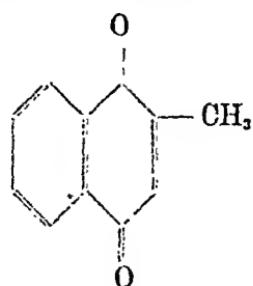
In recent years considerable attention has been given to the study of the effect of certain naturally occurring pigments and redox systems on the respiration of animal and other tissue, and a considerable literature has grown up around this problem. It suffices here to recall the work done on pyocyanine, toxoflavine, echinochrome, hallachrome, and some naphtoquinones like juglon and lawson. We cannot here discuss in detail all the interesting facts which have been brought to light in this field. A good review of the whole subject has been given recently by STERN (1939), and the reader is referred to this standard work for all references. However, much still remains to be done in this field; it is seen from a survey of the literature that only a few scattered substances have been investigated so far. In the present note a report is given on certain results obtained some time ago by the present writer in the study of substances, the investigation of which does not seem to have been reported in the literature, though for some of them the desirability of such an investigation has already been pointed out.

The first of these compounds is phthiocol, the yellow pigment of *Bact. tuberculosis*:

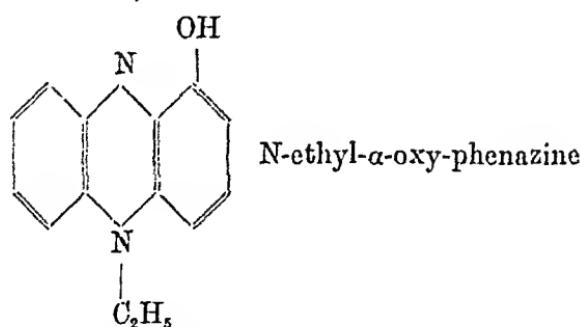
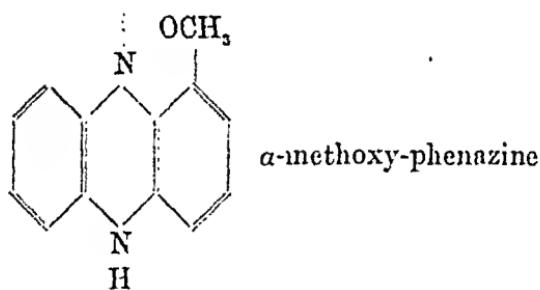
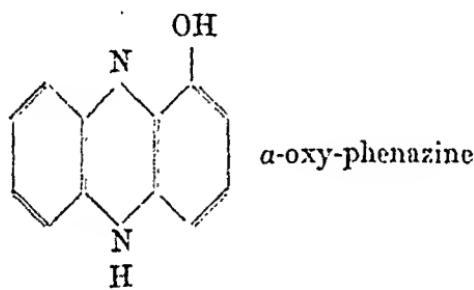


for which no respiration experiments have been carried out so far.

Similar work was then done on 2-methyl-1,4-naphtoquinone, which is not known to occur as a natural pigment in living cells, but is known for its vitamin K activity:



The second class of substances studied in this paper is formed by some compounds closely related to pyocyanine, namely the following:



Pyocyanine itself is N-methyl- $\alpha$ -oxy-phenazine.

Finally we have studied some anthocyanins in their effect on tissue respiration, namely cyanine, which is a diglucoside of the anthocyanidine cyanidine; peonine, the diglucoside of peonidine; and finally malvine, which derives from syringidine and contains two molecules of glucose. The complicated structural formulae of these compounds are omitted here. For anthocyanins and anthocyanidines no respiration experiments seem to have been carried out so far. Their redox potentials have not been determined, and practically nothing is known about their proper biological function. REICHEL states that anthocyanins act as hydrogen acceptors in THUNBERG experiments with purified liver aldehyde hydrase (REICHEL (1937), REICHEL and KÖHLE (1935)), but this hitherto rather isolated observation does not lead us much further towards an understanding of the physiological functions of these compounds. Nevertheless it seems of interest to carry out some respiration experiments with these substances and some results in this direction will be reported in the present paper. Unfortunately work on anthocyanidines could not be included in this note, but it is hoped to return to this subject later on.

### Experimental Part.

*Biological material.* We have worked with kidney, liver and testis tissue from rats. The animals were from 4 to 8 months old, and had been kept on a diet believed to be sufficient in all respects. The animals were killed by decapitation and the organs to be investigated were removed immediately after death.

*Substrates.* Of the substrates investigated the naphtoquinones and the phenazine derivatives were synthesised by the present writer by known methods (see STERN, 1939, for literature). The anthocyanins were obtained from the laboratory of Prof. P. KARRER in Zürich, and the writer is glad to express his best thanks for this valuable gift.

*Experimental procedure.* The Barcroft-Warburg manometric method was employed. The tissue, either in a finely divided state or in the form of slices, was placed in the main chamber of a 10 ml respiration vessel together with 2 ml of Ringer's solution. The central chamber contained 0.2 ml of 5 % KOH for absorption of carbon dioxide. The side chamber contained an aqueous solution or suspension of the substance, the effect of which on tissue respiration was to be examined. We have used in all our experiments solutions or suspensions which were M/25, M/50 and M/100 in the substance to be investigated. However, we quote below only the results for the M/50 solution, the results in the two other cases being quite similar from the qualitative point of view. The thermostat was kept at 38°C. After equilibration for 20 minutes the oxygen con-

sumption was measured for a first period of usually 20 minutes. The solution in the side vessel was then tipped into the main chamber, and the observations were continued for a second period of equal length. This method suffers admittedly from the defect that the partial pressure of carbon dioxide is held at approximately zero, which is a rather unphysiological condition. The great advantage of the method is its extreme simplicity and the identity of the tissue fragments both in the experiment itself and in the controls. It has previously been successfully applied by FRIEDHEIM (1934) in his work on the effect of pyocyanine on the respiration of normal and tumour tissue. The tissue fragments were always introduced into the previously warmed respiration vessel within a period of less than 6 minutes from the death of the animal. The oxygen consumption was found to be strictly proportional to the time for at least 75 minutes. The deviations to be expected with exclusion of bacterial growth are in the direction of decreasing oxygen consumption. Hence these deviations cannot exaggerate any observed increase in respiration.

As Ringer's solution we used both ordinary bicarbonate Ringer and phosphate Ringer. In the experiments in which sugar was added a concentration of 0.2 % glucose was used.

*Results.* To avoid superfluous and tedious tabulation of numerical details we have expressed all our results in the following form: The most probable values of the oxygen consumption during the first and the second period of observation were calculated in a familiar way by means of a diagram in which oxygen consumption was plotted against time. The difference between these two quantities gives the increase in respiration brought about by the substance under investigation, and was finally expressed in per cent of the oxygen consumption during the first period of observation.

Kidney tissue was used in all our experiments in the form of slices cut by means of a razor blade or a sharp knife. Respiration experiments were carried out in bicarbonate and phosphate Ringer both with and without glucose, but no effect whatever on the oxygen consumption was observed for any of the substances investigated. The numerical details are therefore of no general interest and are hence omitted.

Liver tissue was prepared for the experiments by placing it on a watch glass and carefully mincing it by means of a pair of bent scissors. The results of the respiration experiments were: The anthocyanins were found to be completely without effect. Similarly  $\alpha$ -methoxy-phenazine,  $\alpha$ -oxy-phenazine and phthiocol had no influence whatever on oxygen consumption. 2-methyl-1,4-naphthoquinone was likewise without effect in bicarbonate Ringer, both

with and without glucose. However, in phosphate Ringer a very distinct effect was obtained. As an example we quote the following results of a set of experiments, with 3 respiration vessels:

	Percentage increase in oxygen consumption		
Phosphate Ringer without glucose .....	50	35	45
Phosphate Ringer with glucose .....	100	40	50

Analogous results were obtained for N-ethyl- $\alpha$ -oxy-phenazine. The substance had no effect on the respiration of liver tissue in bicarbonate Ringer with or without glucose. In phosphate Ringer, however, an effect of the same order of magnitude as that for 2-methyl-1,4-naphtoquinone was found. The results of a typical experiment were as follows:

	Percentage increase in oxygen consumption		
Phosphate Ringer without glucose .....	25	30	75
Phosphate Ringer with glucose .....	50	70	55

Testis tissue was prepared for the respiration experiments in the same way as liver tissue. Here all the substances investigated were shown to be without any effect, in spite of all efforts to the contrary. A tabulation of the numerical details is therefore of no general interest and is omitted.

Finally some respiration experiments were carried out with yeast cells. Ordinary baker's yeast, suspended in ordinary Ringer or phosphate Ringer, was employed throughout. Phthiocol was found to have no effect on the respiration of yeast cells.  $\alpha$ -methoxy-phenazine was likewise inactive, while for N-ethyl- $\alpha$ -oxy-phenazine the effect was either zero or very small. For  $\alpha$ -oxy-phenazine a typical experiment was as follows:

	Percentage increase in oxygen consumption		
Ordinary Ringer .....	50	60	55
Phosphate Ringer .....	50	75	45

Similarly we obtained for 2-methyl-1,4-naphtoquinone:

	Percentage increase in oxygen consumption		
Ordinary Ringer .....	60	80	82
Phosphate Ringer .....	50	55	72

Hence for both these compounds the effects observed were very considerable.

### Comments.

An exhaustive treatment of the results presented above is hardly possible at the present time on account of the very complex nature of the processes involved and our present very incomplete knowledge of them. Nevertheless, a brief discussion of a few points may not be out of place.

Phthioeol is a relatively negative redox system, and in our experiments it was found to have no effect on tissue respiration. The potential of 2-methyl-1,4-naphtoquinone does not seem to be known, but it may perhaps not be so different from that of phthioeol. In our experiments it was found to raise the oxygen uptake of liver cells very considerably. In this connection it may be of importance to remember that the redox potential is not the only factor to decide whether a substance can act as carrier; its chemical constitution, for instance, is also of importance.

The inactivity of  $\alpha$ -methoxy-phenazine can probably be well accounted for on the basis of present knowledge of the structure of those phenazine compounds that form reversible redox systems (see STERN (1939), p. 228 et seq.). N-ethyl- $\alpha$ -oxy-phenazine was found to behave very much like pyocyanine itself, as one would probably expect it to do. It is a curious phenomenon that there is no effect except in phosphate Ringer. A somewhat similar observation was previously made by FRIEDHEIM (1934). Like pyocyanine, the substance has no effect on cells with a perfect respiration, i. e. exhibiting no aerobic glycolysis whatsoever (kidney). There are some differences, however, which are perhaps not so readily understood at the present time. According to FRIEDHEIM the presence of glucose is essential for the increase in respiration observed with pyocyanine, but this was certainly not the case in our experiments with its ethyl homologue. Further, pyocyanine was found to increase the oxygen uptake of testis tissue, but N-ethyl- $\alpha$ -oxy-phenazine was inactive in this case.

The other results do not seem to call for much comment at the present time. It would be interesting to continue the work, especially with anthocyanidines, and as already mentioned it is hoped to do so in a later note.

The author is glad to express his best thanks to Prof. R. EGE for his generous support and hospitality.

### Summary.

Certain naphthoquinones, phenazine derivatives and anthocyanins have been subjected to respiration experiments with the BARCROFT-WARBURG technique, using kidney, liver and testis tissue and yeast cells. Some of the results can be fitted into our present picture of these processes, but certain findings appear to be somewhat at variance with those of previous workers in this field.

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## Further Investigations on the Effect of Adenosine Triphosphate and Related Phosphorus Compounds on Isolated Striated Muscle Fibres.

By

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In a previous communication it has been shown that adenosine triphosphate (ATP) in extremely small amounts (approx.  $10^{-5} \mu\text{g}$ ) releases contraction in normal and curarized striated muscle fibres. Furthermore, ATP causes a reversible decrease in birefringence, while inorganic triphosphate initiates contraction without changing this property. The changes in birefringence after application of ATP last considerably longer than the mechanical process of contraction.

In the present investigation the effect of ATP on striated muscle is studied under different conditions. In view of the effect of inosine triphosphate (ITP) in model experiments on myosin solutions, it seemed of interest to examine the effect of this compound on muscle fibres and at the same time to extend the investigation to comprise other phosphorus compounds of high bond energy. To begin with we have examined creatine phosphate and acetyl phosphate.

### Method.

The experiments were carried out on isolated fibres or small bundles of the frog's m. semitendinosus (*Rana esculenta* and *Rana temporaria*). The threshold for release of contraction by the different chemical

stimuli was determined on small fibre bundles (2–8 fibres) which were fixed by their tendon ends in two movable metal clamps on a slide. During preparation and the experiment the fibre was kept in a Ringer solution at 10° containing 6.7 g NaCl, 0.2 g KCl, 0.2 g anhydrous CaCl<sub>2</sub> and 0.2 g glucose per litre. The pH was kept constant at 7.3 by adding a suitable amount of NaHCO<sub>3</sub>, and passing a stream of a CO<sub>2</sub>—O<sub>2</sub> mixture through the Ringer solution. The normal colloid osmotic pressure was attained by supplying the Ringer solution with 3 per cent dextran (GRÖNWALL and INGELMAN 1945). The chemical substances were administered with a fine pipette when the amount of liquid was 0.03 ml, or with a micro-pipette for liquid volumes of

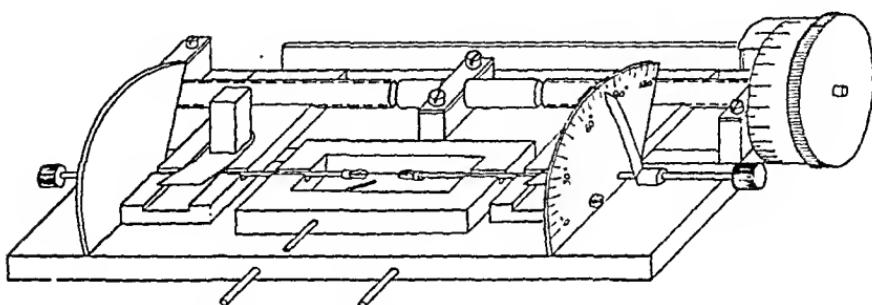


Fig. 1. Muscle chamber provided with an arrangement to turn the fibre 90° for measuring thickness and phase difference in the same plane.

approx.  $6 \times 10^{-3} \mu^3$ . In all cases the effects of chemical stimuli were tested in non curarized and completely curarized fibres and compared with electrical stimulation by single current pulses and tetanic stimuli from a thyratron stimulator. For the experiments on the denervated muscles the sciatic nerves were divided under ether anaesthesia and the animals were kept for some 3 weeks to allow degeneration to proceed to completion.

In measurements of birefringence  $\left(\frac{I'}{d}\right)$  only isolated fibres were used, their tendon ends being held by two pairs of silver tweezers in a chamber at constant temperature (Fig. 1). Phase difference ( $I'$ ) was determined with a Babinet compensator as in former experiments (BUCHTHAL and KNAPPEIS 1938).

When determining fibre thickness (d) we have hitherto measured the diameter by means of an eye-piece micrometer with movable cobweb in a plane perpendicular to that used for determination of phase difference. In the course of other experiments we found considerable deviations in the circular cross section of the fibres, which may introduce serious errors in the absolute values of birefringence. To eliminate this the fibre was rotated through 90° by turning the two pairs of tweezers. The fibre diameter was thus measured in the same plane as that used for determining phase difference.

## Preparation of substances.

*Adenosine triphosphate (ATP).* The ATP was prepared from rabbit muscle as the Ba salt by the method of D. M. NEEDHAM (1942), and as the neutral or acid Ba salt and the Ca salt by the method of KERR (1941). No Ba salt and only Ba-free reagents were used in the preparation of Ca ATP.

A solution of the Na ATP was prepared from the Ba salt by addition of the calculated amount of sodium sulphate to the Ba salt either suspended in water or dissolved in dilute hydrochloric acid. The Ca ATP was converted into the Na salt by the addition of the calculated amount of sodium oxalate to the solution of Ca ATP in dilute hydrochloric acid. Both the isolated Ba and Ca salts and the final Na ATP solutions were analysed for N and for total and 7' P according to the method of FISKE and SUBBAROW (1925) in the modification of SCHEEL (1936). The purity of each substance was at least 98—99 per cent.

*Inosine triphosphate (ITP).* The substance was prepared as the Ba salt from Ba ATP according to KLEINZELLER (1942).

A solution of Na ITP was prepared by addition of the calculated amount of sodium sulphate to the Ba salt suspended in water. Analysis of both the Ba salt and the Na ITP for N and total and 7'P indicated a purity of at least 98—99 per cent.

*Creatine phosphate* was prepared by the method of ZEILE (1938) by phosphorylation of creatine with  $\text{POCl}_3$ . The isolated Ca salt was first purified according to ZEILE and then converted into the Ba salt, which was recrystallized repeatedly from water-methanol according to DEUTSCH et al. (1938). Analysis: P, 8.4 per cent, N, 11.3 per cent;  $\text{C}_4\text{H}_8\text{O}_5\text{N}_3\text{PBa} \cdot \text{H}_2\text{O}$  requires: P, 8.5 per cent, N, 11.5 per cent. A solution of Na creatine phosphate was prepared by precipitation of Ba with the calculated amount of sodium sulphate from the solution of the Ba salt in water.

*Acetyl phosphate.* The substance was prepared according to LYNEN (1940) as the silver salt. (P, 8.9 per cent; calc. for  $\text{C}_2\text{H}_3\text{O}_5\text{PAg}_2$ : P, 8.8 per cent). A solution of sodium salt was prepared by addition of the calculated amount of NaCl to the suspension of the silver acetyl phosphate in water.

*Adenosine.* The substance was prepared from yeast nucleic acid by the method of BREDERECK (1938) and recrystallized repeatedly from water. (N, 26.2 per cent; calc. for  $\text{C}_{10}\text{H}_{15}\text{O}_4\text{N}_5$ : N, 26.2 per cent).

All substances were applied *iso-osmotically* by replacing part of the NaCl + water in the Ringer solution by a corresponding amount of the substance dissolved in water, the solution being adjusted to pH 7.3.

## Results.

### 1. Adenosine triphosphate.

ATP applied to striated muscle showed the same effect with respect to release of contraction and changes in birefringence as described in our previous paper (1944), regardless of the method.

of preparation of the Ba salt or of the resulting Na ATP solution. Since solutions containing Ba ions were very active when applied to heart muscle (unpublished experiments of DEUTSCH and LUNDIN), we thought it necessary to investigate the effect of (1) Ba salts on skeletal muscle, and (2) an ATP solution prepared with complete exclusion of Ba ions during all phases of the preparation.

$BaCl_2$  in the concentrations investigated (0.01—0.002 M) has no effect on striated muscle fibres. Na ATP from the Ca salt prepared according to KERR, but without the use of barium salts, acts like all other ATP preparations on contraction and birefringence with a threshold value at a concentration of  $0.72 \times 10^{-6}$  mol/ml.

In work on myosin, calcium and magnesium ions have been shown to play an important part in the interaction between myosin and ATP. In muscle the threshold for ATP increases considerably with increasing concentration of *calcium ions* applied isotonically. The threshold for ATP is about 50 times higher in the absence of Ca or if it is present in excess (0.009 M  $CaCl_2 \cdot 6 H_2O$ ;  $\frac{K}{Ca} = 0.1$ ), corresponding to 5 times the Ca concentration in normal Ringer solution.

While there is an optimum concentration for Ca, the presence of magnesium ions increases the threshold value for ATP and for electrical stimulation. With 0.01 M  $MgCl_2$  applied iso-osmotically in Ringer solution the threshold is 10 times normal, while it is still higher with 0.05 M  $MgCl_2$ .

## 2. Inosine triphosphate.

Inosine triphosphate is more active than ATP in releasing contraction, the threshold concentration being  $0.25 \times 10^{-6}$  mol/ml. Larger amounts initiate repetitive activity of long duration. Birefringence remains constant even when the substance is applied in a thirty times higher concentration than its threshold for release of contraction.

## 3. Creatine phosphate.

It was of special interest to compare creatine phosphate and ATP in their effects on the isolated muscle fibre, since the break-

down of these two substances occurs nearest in time to the contraction process. Creatine phosphate neither releases contraction nor affects birefringence (concentration applied  $7.2 \times 10^{-6}$  mol/ml).

When applied simultaneously with small amounts of ATP, creatine phosphate increases the effect of the former, and can release contraction anew in a muscle fibre in which contractions evoked by ATP have already ceased. This effect is probably due to the regeneration of ATP by the added creatine phosphate.

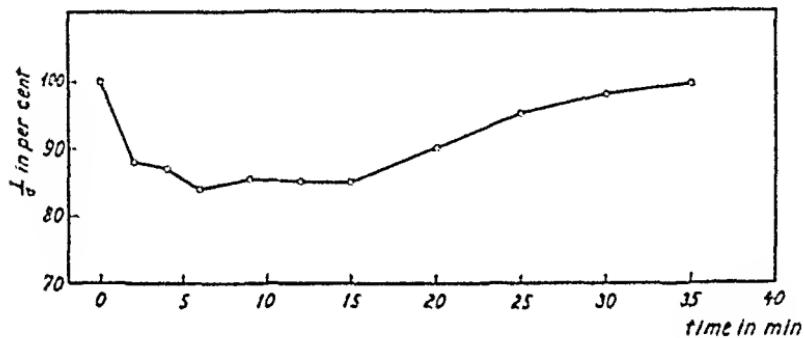


Fig. 2. Birefringence as function of time after application of creatine phosphate + adenylic acid ( $7.2 \times 10^{-6}$  mol/ml). Substances applied at 0 min.

*Creatine phosphate + adenylic acid* releases contraction (threshold  $3.6 \times 10^{-6}$  mol/ml) and causes a reversible fall in birefringence of about 15 per cent, in concentrations of  $7.2 \times 10^{-6}$  mol/ml. But whereas with ATP birefringence attains its minimum after 2 minutes and is completely restituted within 20 minutes, after application of creatine phosphate + adenylic acid restitution begins after 12—15 minutes and is complete only after about 30 minutes (Fig. 2).

#### 4. Acetylcholine-adenosine triphosphate.

According to FELDBERG and MANN (1945) ATP is necessary for the resynthesis of acetylcholine in cell-free brain extracts. We have sought for evidence for such an interaction in frogs muscle, and investigated whether the local distribution of ATP in muscle points to a close relationship of this substance with the function of the motor end plate. The pelvic part of the frog's sartorius muscle (1/5th of its length) has been shown to be entirely free

from nervous elements (MURNAY and NACHMANSOHN (1938), PÉZARD and MAY (1937)). Phosphate analysis of this part of the muscle reveals, however, no differences in ATP content in comparison with the rest of the muscle. In search for a common link between acetylcholine and ATP we have examined another high-energy phosphate, acetyl phosphate, the presence of which has recently been demonstrated in living tissues by LIPPMANN (1942). This substance, however, even in high concentrations (up to  $14.4 \times 10^{-6}$  mol/ml) has no effect whatever on the contractility or birefringence of non-curarized or curarized muscle fibres.

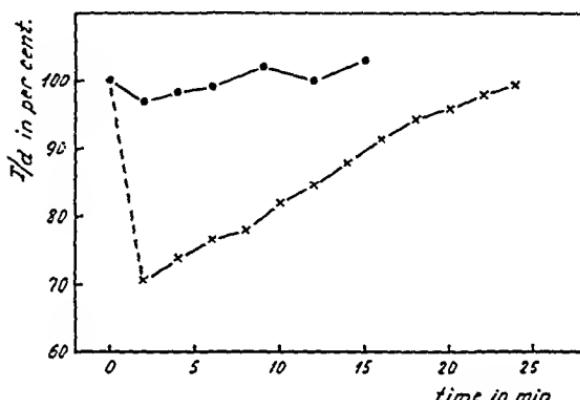


Fig. 3. Birefringence as function of time.

$\times-x-x-x-x$  application of ATP ( $7.2 \times 10^{-6}$  mol/ml)

$\bullet-\bullet-\bullet-\bullet$  application of ATP ( $7.2 \times 10^{-6}$  mol/ml) after previous treatment with acetylcholine ( $10 \mu\text{g}/\text{ml}$ ). Substances applied at 0 min.

Acetylcholine, on the other hand, can interfere with the action of ATP in striated muscle. It has previously been observed that ATP sensitizes amphibian and mammalian muscle for subsequent application of acetylcholine (BUCHTHAL and KAHLSON (1944), BUCHTHAL and FOLKOW (1944)). This sensitization is, however, localized to the contractile substance, as the response of curarized muscle to direct electrical stimulation is likewise considerably increased after previous application of ATP. When a muscle fibre is treated with minute amounts of acetylcholine ( $10 \mu\text{g}/\text{ml}$ ), this substance eliminates the change in birefringence otherwise present after application of ATP (Fig. 3). In denervated amphibian muscle, just as in mammalian muscle (BUCHTHAL and KAHLSON (1946)), previous treatment with acetylcholine even abolishes release of contraction by ATP.

*Denervated amphibian muscle* has a reduced sensitivity towards ATP. A concentration producing a long-lasting repetitive activity in curarized and non-denervated muscle fibres, evokes only a short tetanic contraction in denervated muscle. Birefringence here remains constant after application of ATP.

### 5. Adenosine.

Adenosine, like adenylic acid, has no effect on striated muscle (concentration examined  $7.2-45 \times 10^{-6}$  mol/ml), but applied together with pyrophosphate it lowers the threshold of the latter and at the same time changes the type of contraction from contracture-like to repetitive twitch-like activity. While pyrophosphate causes irreversible changes in birefringence the application of adenosine + pyrophosphate leaves the latter unchanged. It may be worth mentioning that aneurin + pyrophosphate applied to the muscle have the same effect as adenosine + pyrophosphate, while aneurin without pyrophosphate has no effect whatsoever. It is tempting to assume that the aneurin + pyrophosphate effect is due to the formation of cocarboxylase.

### Discussion.

The action of adenosine triphosphate on striated muscle fibres, as demonstrated in a previous communication, is twofold, viz. it releases contraction and initiates reversible changes in birefringence. In view of the time relations between mechanical processes and changes in birefringence, the latter are considered as an expression of restitutational processes in the contractile proteins. The results are interpreted by assuming ATP to be the normal agent of contraction, initiating a *discharge* of contractile proteins, and the primary link in the transfer of energy for restitution, *i. e. recharge* of contractile proteins. The effect is highly specific for ATP and only adenosine diphosphate and adenylic acid + pyrophosphate have the same mode of action. The latter substances, however, can be considered as ATP precursors.

Inosine triphosphate is more active in releasing contraction than ATP but is without effect on birefringence. Contraction can also be evoked by inorganic triphosphate, indicating that the triphosphate part of the ATP molecule is sufficient for its

release.<sup>1</sup> On the other hand, the changes in birefringence caused by ATP are highly specific.

This implies that restitution does not occur after application of triphosphate or inosine triphosphate, although the latter only differs from ATP in that an NH<sub>2</sub> group is replaced by OH. In fact it is found that triphosphate only acts once when administered by micro-application, while the effect of ATP can be repeated several times.

The investigation of the effect of creatine phosphate was of special interest, since the present experiments allow a direct comparison of the two energy-supplying compounds, the breakdown of which occurs nearest in time to the contraction proper. Although experiments on muscle extract favour the view that the breakdown of ATP is the primary reaction, other investigators (LIPMANN (1941)) consider the question still open, as processes occurring in unorganised systems such as muscle extract cannot be compared with energy transfer in the intact muscle. The completely negative results with creatine phosphate regarding both release of contraction and changes in birefringence, decide in favour of ATP as the agent of contraction and the immediate source of energy for the recharge of the contractile protein. ATP can be substituted in its effect on muscle by creatine phosphate + adenylic acid, a fact which is in agreement with results from work on muscle extracts, viz. that the hydrolytic breakdown of creatine phosphate is coupled to the intermediate formation of ATP.

### Summary.

1. In agreement with previous results it was found that ATP prepared in different ways initiates contraction and changes birefringence when applied in minute amounts to isolated striated muscle fibres of the frog. ATP is evenly distributed in the muscle and not localized specially in the region of the motor end plate.

2. Lack or excess of calcium just as excess of magnesium increase the threshold value for ATP.

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<sup>1</sup> We wish to correct an erroneous statement in the discussion of our previous paper (1944, p. 285). Evidence was presented that the triphosphate part of the ATP molecule was energetically and structurally sufficient to release contraction, and not, as stated, both the triphosphate and nucleotide parts of the molecule.

3. Adenosine alone is without effect, it changes the contracture-like effect of pyrophosphate to a normal tetanus-like contraction.

4. Inosine triphosphate, thrice as active as ATP in initiating contraction is without effect on birefringence.

5. Creatine phosphate has no effect on striated muscle, thus establishing the breakdown of ATP as the reaction nearest in time to contraction. Creatine phosphate + adenylic acid can replace ATP in the release of contraction and causes a reversible fall in birefringence. The time of restitution, however, is considerably longer than with ATP.

6. Acetyl phosphate is without effect.

7. Acetylcholine abolishes the effect of ATP on birefringence while it does not affect release of contraction. In denervated muscle previous application of acetylcholine prevents release of contraction by ATP. Contraction in denervated muscle is not accompanied by the slow changes in birefringence.

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*Added in the proof:* A paper by TORDA and WOLFF (1946) came recently to our knowledge in which it is discussed, how far a release of contraction by ATP may be due to the removal of intracellular calcium by the liberation of phosphate. In this connection it can be of interest to mention our experiments on the effect of sodium citrate. The threshold concentration of citrate in releasing contraction is higher than that of ATP.

Previous treatment of a muscle with strong concentrations of citrate ( $3 \times 10^{-5}$  mol/ml) does not abolish the sensitivity to ATP. No decrease in birefringence occurs after application of citrate ( $3.6 \times 10^{-6}$  mol/ml), while it inhibits the decrease in birefringence caused by ATP. These experiments give no support to the assumption that the action of ATP is due to the removal of intramuscular calcium.

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# Rate of Renewal of Ribo- and Desoxyribo Nucleic Acids.

By

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Enzymic processes coupled with phosphorylation take often place at a remarkably rapid rate. A large percentage of the molecules of many of the acid-soluble phosphorus compounds and, to a minor extent, also those of phosphatides present in the liver and some other organs are renewed within a short time. This is demonstrated by the observation that shortly after the administration of  $^{32}\text{P}$  these molecules are found to contain labelled phosphate.

That the presence of  $^{32}\text{P}$  in the molecules of organic phosphorus compounds indicates an enzymic synthesis of such molecules is most strikingly demonstrated by a recent experiment of CHAIKOFF and his associates (1942). These authors have shown that labelled phosphatides are formed when surviving liver slices are shaken with bicarbonate Ringer solution containing labelled phosphate, that this formation is, however, impaired in the absence of oxygen, and homogenized liver tissue completely loses its ability to incorporate  $^{32}\text{P}$  into the phosphatide molecule. A non-enzymic process could hardly be dependent on the intactness of the tissue cells.

In contradistinction to the above mentioned compounds, as found in previous (HEVESY and OTTESEN 1943, AHLSTRÖM, EULER and HEVESY 1944, BRUES, TRACY and COHN 1944) and in the present investigations, desoxyribo nucleic acid molecules present in the liver are renewed at a very slow rate only. This result falls in line with the view that desoxyribo nucleic acid

is present in the nuclei of the cells and is involved in the process of cell division. As the mitotic process in the liver of a fully grown rat takes place at a very slow rate only, the low rate of renewal of desoxyribo nucleic acid in the fully grown liver is in no way surprising, neither is so the much higher rate of renewal observed in the liver of the only few days old rat. The rate of formation of new desoxyribo nucleic acid molecules present in the liver of 3 to 4 days old rats was found to be about 20 times that of the corresponding figure in outgrown rats (AHLSTRÖM, EULER and HEVESY 1944).

In contradistinction to the desoxyribo nucleic acid, a large part of ribo nucleic acid is located in the cytoplasm and, according to the view developed by CASPERSSON (1940), is involved in protein synthesis. As such a synthesis takes place at a marked rate in the liver, the rate of renewal of the ribo nucleic acid can be expected to be larger than the rate of renewal of desoxyribo nucleic acid in this organ. The present note contains the results of experiments in which the rate of renewal of both types of nucleic acid was determined, viz. in the liver, the spleen, and the intestinal mucosa of the rat and also in the total rat body. The methods applied in the isolation of the nucleic acids will shortly be published by one of the authors.

### Results.

If we assume the free phosphate to be built into the nucleic acid molecules without the formation of an intermediary phosphorus compound of comparatively long life, the ratio of the specific activity of the nuclei acid P and the free P is a measure of the rate of formation of new nucleic acid molecules and thus of the extent of renewal of such molecules. If, previous to the formation of labelled nucleic acid molecules, labelled precursors would be built up at a rate slower than the rate of formation of new nucleic acid molecules, the ratio of the specific activity of the nucleic acid P and that of the free P would no longer be a proper measure of the rate of renewal of the nucleic acid. In the latter case, namely, during part of or possibly throughout the whole experiment new nucleic acid molecules would be built up without participation of  $^{32}\text{P}$ . The participation of such labelled intermediary compounds of a very long life in the formation of desoxyribo nucleic acid in the liver is, however, highly improbable in view of the comparatively short lifetime of most of the

acid-soluble phosphorus compounds and the very long lifetime of desoxyribo nucleic acid molecules present in the liver.

In our experiments the specific activity of the nucleic acid P is compared with the specific activity of the free P at the end of the experiment. As the specific activity of the free P changes throughout the experiment, the specific activity of the nucleic acid P at the end of the experiment should, however, be compared with the mean specific activity of the free P during the experiment. As the liver takes up  $^{32}\text{P}$  at a very rapid rate, and its free  $^{32}\text{P}$  content culminates within the first 2 hours, the end value and the average value of the specific activity of the free liver P do not differ essentially, the average value being about 5 per cent lower than the end value (AHLSTRÖM, EULER and HEVESY 1944). In the case of the spleen the corresponding figure is about 25, and even larger differences are found in the case of the intestinal mucosa. The figures of columns 2 and 3 of Table 1 should therefore be multiplied with 1.05 in the case of the liver, for example, to obtain a correct value for the amount of the rate of renewal of the desoxyribo nucleic acid present in the liver. In the figures of Table 1 we have not introduced this correction, as we are mainly interested in the relative rate of renewal of the desoxyribo and ribo nucleic acids.

A further point which was not considered is the repeated renewal of the same molecule during the experiment. In view of the percentage renewal amounting in our experiment to few per cent of less only, the probability of repeated renewal of a molecule during the experiment is small, though the problem whether all nucleic acid molecules present in the liver have the same chance of being renewed or some of them are situated in preferential districts of the cell and are thus renewed at a more rapid rate is yet unsolved.

Table 1 contains the results of an experiment in which three rats in nitrogen-equilibrium weighing 252, 182 and 215 g were injected subcutaneously with respectively 7.5, 6.6 and 5.6 micro-curies of  $^{32}\text{P}$  per 100 g body weight.

After 2 hours the animals were killed and the organs prepared according to a method which will soon be published by one of the authors.

As recorded in Table 1, the rate of renewal of ribo nucleic acid in the liver is as much as 33 times larger than the rate of renewal of desoxyribose nucleic acid. In spite of the finding that

ribo nucleic acid is renewed at an even larger rate in the spleen and the intestinal mucosa than in the liver, the ratio of the rate of renewal of ribo and desoxyribo nucleic acids in these organs is only 3 and 2, respectively. This low ratio is due to the comparatively high rate of renewal of desoxyribo nucleic acid in these organs. From the above figures it follows that the rate of renewal of both types of nucleic acid is highest in the intestinal mucosa and in the spleen.

Table 1.

*Ratio of the rate of renewal of the ribo nucleic acid and desoxyribo nucleic acid in the organs of the rat in the course of 2 hours.*

Organ	Percentage ratio of the specific activity of the nucleic acid P and that of the free P		Ratio of the rate of renewal of ribo and desoxyribo nucleic acid
	Ribo	Desoxyribo	
	nucleic acid		
Liver . . . . .	3.8; 3.6	0.12; 0.09	33
Spleen . . . . .	3.1; 10.2	2.2; 2.2	3
Intestine . . . . .	7.1; 4.1	3.4; 2.3	2

The specific activity of both the desoxyribo and the ribo nucleic acid P extracted from the rat liver was determined by BRUES, TRACY and COHN (1944) in experiments lasting 3 to 8 days. In these experiments the ribo nucleic acid P was found to be only 5 to 6 times as active as the desoxyribose nucleic acid P. The discrepancy between these figures and those obtained by us may be due, at least in part, to the much longer duration of the experiment.

#### Specific Activity of the Nucleic Acid Phosphorus Extracted from the Total Rat.

In another experiment the specific activity of both the total desoxyribo nucleic acid P and the total ribo nucleic acid P extracted from a rat weighing 194 g was determined. The activity of labelled sodium phosphate amounted to 8.1 microcuries per 100 g animal weight. The time of the experiment was 2 hours. The results of this experiment are seen in Table 2.

Table 2.

*Specific activity of the nucleic acid P extracted from the total rat compared with the corresponding values for liver, spleen, and intestinal mucosa. The value for the specific activity of the total rat ribo P is assumed to be = 100.*

S a m p l e	Specific activity		
	Ribose	Desoxyribose	Free P
	nucleic acid		
Total rat . . . . .	100	60	
Liver . . . . .	164	4.4	5100
Spleen . . . . .	292	63	2850
Intestine . . . . .	112	63	2770

As shown in Table 2, the specific activity of the average nucleic acid P of the rat is almost identical with the corresponding value of the ribo and desoxyribo nucleic acid, respectively extracted from the intestine.

The interpretation of the significance of the specific activity figures obtained for the total rat encounters some difficulties, as the specific activity of the free P utilized in the formation of the labelled nucleic acid molecules is unknown. If the specific activity of the free P utilized in building up the average body nucleic acid would correspond to the specific activity of the free liver P, the percentage "rate of renewal" of the body ribo and desoxyribo nucleic acids would be 2.0 and 1.2, respectively. If the specific activity of the free P utilized in building up the average nucleic acid of the organism would correspond to the specific activity of the free intestinal P, larger figures, *i. e.* 3.6 and 2.2, respectively, would be obtained.

When calculating the last mentioned figures we compared the specific activity of the nucleic acid P at the end of the experiment with the specific activity of the free intestinal P at the end of the experiment. Correctly we should have considered the mean value of the specific activity of the three intestinal P prevailing during the experiment. The mean value of this magnitude is about almost half of its end value, we have therefore to multiply the figures mentioned above (3.6 and 2.2 respectively) with about 2 to obtain an approximate value of the per-

centage renewal of the ribo- resp. desoxyribo nucleic acid in the course of 2 hours.

It is improbable that a so highly active free phosphate is utilized in the building up of the nucleic acid molecules as found in a 2 hours experiment in the liver. Liver and kidneys have a privileged position concerning the rate of intrusion of phosphate. The amount of nucleic acid present in the liver and the kidneys makes out, furthermore, only a small percentage of the total nucleic acid content of the organism. It is much more probable that free P of similar specific activity as found in the intestine is applied in the building up of the labelled nucleic acid molecules. In fact, the amount of nucleic acid present in the mucosa of the digestive tract makes out a large percentage of the body nucleic acid. While the body nucleic acid contains also slightly radioactive fractions, viz. those originating from the liver, the kidneys, and the brain and fractions of restricted radioactivity originating from the muscles (HEVESY and OTTESEN 1943), it contains also fractions of higher activity than found in the intestinal mucosa, viz. those originating from the bone marrow, the thymus and lymph nodes (ANDREASEN and OTTESEN 1944, 1945). The lymphocytes secreted into the organism can also be expected to contain pronouncedly active nucleic acid. This makes it understandable that the rate of renewal of the average body nucleic acid corresponds to about the rate of renewal of the intestinal nucleic acid and is thus quite pronounced for both types of nucleic acid in contradistinction to the rate of renewal found in the liver, which is very low in the case of desoxyribo nucleic acid and appreciably higher in the case of ribose nucleic acid.

### Discussion.

The mitotic figure of the fully grown liver is unknown. In hemectomized livers of quite young rats (50 g) figures of 1—2 per cent are recorded (MARSHAK and WALKER 1945). Even if the formation of labelled desoxyribo nucleic acid molecules in the fully grown rat is in direct connection with mitotic processes, the mitotic figure can not be as high as 0.1 per cent (a mitotic division lasts about 1—2 hours), since in some phases of the mitotic process nucleic acid disappears, in others accumulates, as demonstrated by CASPERSSON (1940). When comparing, furthermore, the number of labelled desoxyribo nucleic

acid molecules formed in growing Jensen sarcomata of the rat with the increase in the desoxyribo nucleic acid content due to growth, the former figure was found to be higher than the latter (EULER and HEVESY 1944) and a similar result was obtained when comparing the formation of labelled desoxyribo nucleic acid molecules in 3 days old rats with the increase in the desoxyribo nucleic acid content due to growth of the liver.

In view of the high desoxyribo nucleic acid content of the lymphocytes and because they are partly produced in the spleen, the comparatively high rate of turnover of desoxyribo nucleic acid in the spleen is in agreement with our expectance.

It is more difficult to interpret the high figures found for the rate of renewal of desoxyribo nucleic acid in the intestinal mucosa. The intestinal mucosa is exposed to very hard tear, cells are destroyed at a large scale and new ones are formed. It is, however, very problematic whether this process alone can account for a so high rate of renewal of the desoxyribo nucleic acid as found in this organ.

If we accept the view put forward by CASPERSSON, the high rate of renewal of ribo nucleic acid is in no way surprising. That the high figures found for the rate of renewal of ribo nucleic acid in the intestine, the spleen and the liver is just what we would expect in view of the importance of these organs in protein metabolism. The incorporation of labelled sulfur into protein sulfur is found to be higher in the intestine than in any other organ (TARVER and SCHMIDT 1942) and the  $^{15}\text{N}$  content of the proteins isolated from the intestinal wall of the rat after administration of isotopic L(—)-leucine is larger than the corresponding value for any other organ investigated. Somewhat smaller values for the  $^{15}\text{N}$  content of the proteins isolated from the spleen were found, and still smaller values for the  $^{15}\text{N}$  of the proteins isolated from the liver (SCHOENHEIMER, RATNER and RITTENBERG 1939). The rate of formation of ribose nucleic acid in these three organs diminishes in the same sequence.

If we want to state, not as above the percentage, but the amount of nucleic acid formed during the experiment, we must know the nucleic acid content of the organs of the rat and of the total rat.

Some preliminary figures for the total nucleotide P of the liver, spleen, intestine and total rat and also some preliminary figures for the share of polydesosine and polyribo nucleotides in the total

nucleotides is seen in Table 3. The method applied in obtaining these figures and more accurate data will be shortly published by one of the authors.

Table 3.

*Polydesose nucleotide phosphorus and Polyribo nucleotide phosphorus content of some organs and of the total rat.*

	Approximate share of polydesose nucleotides in the total nucleotides	g nucleotide P per 100 g dry weight	g polydesose nucleotide P per 100 g dry weight	g polyribo nucleotide P per 100 g dry weight
Total rat . . . . .	45—50 %	0.232	0.11	0.12
Liver . . . . .	35 %	0.350	0.12	0.23
Spleen . . . . .	75 %	0.643	0.48	0.16
Intestine . . . . .	57 %	0.669	0.38	0.29

Assuming the percentage renewal of the polydesose nucleic acid of the total rat in the course of 2 hours to be 4 (cf. p. 339) and the fresh weight of the rat to amount to five times its dry weight, in a 200 g rat in the course of 2 hours about 2 mg polydesose nucleotide P will be renewed. The corresponding figure for the polyribo nucleotide P works out to be 3. In the total rat the turnover rate of the 2 types of polynucleotides does thus not differ very appreciably.

A very different result is obtained when comparing the amount of polydesose and polyribo nucleotide phosphorus renewed in the liver. The figures work out, assuming the liver to weigh 6 g, to be 0.0017 mg and 0.094 mg respectively. Fiftyfive times more polydesose nucleotide than ribo-nucleotide gets thus renewed in the liver during the same time.

Assuming the spleen to weigh 0.8 g, both the amount of polydesose nucleotide P and that of polydesose nucleotide P formed and still present in the spleen works out to be about 20 microgram.

How far the rate of enzymic replacement of other constituents of the nucleic acid molecule, for example that of the pyridine and pyrimidine groups, takes place at a similar rate as the enzymic replacement of phosphorus is not yet elucidated.

### Summary.

Labelled sodium phosphate is administered to rats and after the lapse of 2 hours the specific activity of the ribo-nucleic acid phosphorus and that of the desoxyribo-nucleic acid phosphorus determined.

In the liver the specific activity of the ribo-nucleic acid P is found to be 33 times larger than the specific activity of the desoxyribo-nucleic acid P. In the course of 2 hours about 0.1 and 3.3 per cent respectively of these compounds were found to be renewed.

In the intestine and in the spleen in which the specific activity of the desoxyribo-nucleic acid is found to be about 20 times larger than the corresponding value in the liver, the specific activity of the ribo-nucleic acid phosphorus is only 2 to 3 times larger than the corresponding value of the desoxyribo-nucleic acid phosphorus.

The ribo- and the desoxyribo-nucleic acid phosphorus extracted from the total rat have a very similar specific activity to the corresponding phosphorus extracted from the intestine. In the total rat the difference in the rate of renewal of the two types of nucleic acid is not very pronounced. In a rat weighing 200 g approximately about 2 mg desoxyribo-phosphorus and 3 mg ribo-nucleic acid phosphorus are turned over in the course of 2 hours.

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## On the Purification of the Thiamin-Inactivating Fish Factor II.

By

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A method was recently described for the tenfold purification of the thiamin-inactivating fish factor (ÅGREN 1945 a). By means of this method definite proofs were obtained that the thiamin-splitting principle was not a haemin protein. On the other hand, the purified solution of the factor was always yellow-coloured and it had to be settled whether this circumstance was a mere coincidence. The present paper reports the further purification of the factor to a colour-free substance.

### Experimental.

The enzyme was prepared from the viscera of ide according to the above mentioned method (ÅGREN 1945 a) and subsequently stored at — 15° C. The viscera were collected under the same precautions as previously described (LIECK and ÅGREN 1944). The activity of the factor was ascertained by a method based on the diazo reaction of PREBLUDA and McCOLLUM. This method was a modification of the procedure described by MELNICK and FIELD 1939 (cf. LIECK and ÅGREN 1944). The activity was expressed in units as previously defined (ÅGREN 1945 a).

### Results.

The activities of the solutions of the factor purified according to the above-mentioned method were rather unstable, even when the material was stored at — 15° C. Possible means of

further purification seemed, however, to be few. Finally a separation of impurities was attained by means of cataphoresis. As previously stated (ÅGREN 1945 a), a preliminary cataphoretic analysis of the purified solutions of the factor usually revealed the presence of three fractions, all of which seemed to move parallel at different pH values. A further analysis now showed that a separation could be obtained by cataphoresis at pH 4.7.

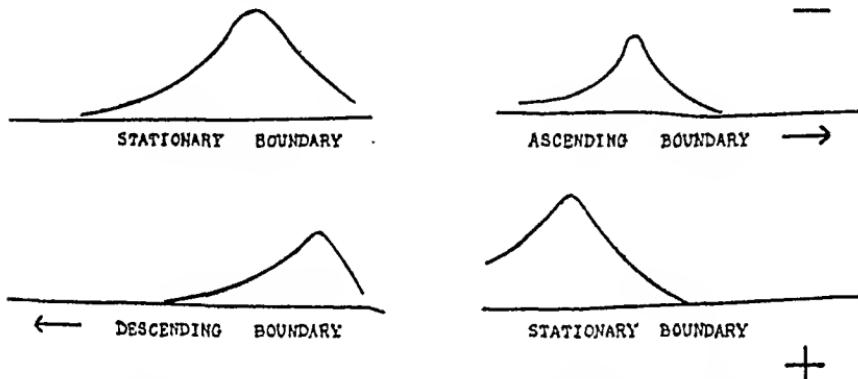


Fig. 1. Electrophoretic diagram obtained from an extract of the purified factor. For experimental conditions see the text.

Visual observations and photographic records obtained with the optical arrangements, described by SVENSSON (1939), disclosed the presence of two components of different electrochemical behaviour (Fig. 1). The activity was associated with the small fraction which exhibited a slow cathodic mobility, while a larger fraction was rather stationary throughout. The results were easily reproducible with extracts of ide (cf. discussion).

Solutions for cataphoretic purification were prepared on a large scale in the following way. Samples of 1–2 kg of viscera (liver, spleen, intestines and gills) from about 30 kg of ide were purified as previously described (ÅGREN 1945 a). The purified solutions usually contained about 2 mg of protein nitrogen and 3–4 units of activity per ml of solution. 40 ml of this solution was dialyzed in a cellophane tube ( $\varnothing = 27$  mm) at  $0^\circ\text{C}$ . for 1 hour against distilled water and for 2 hours against the cataphoretic buffer on a cradle dialysis apparatus. The contents of the cellophane tube were then centrifuged from a precipitate which formed during the dialysis. The centrifugate was dialyzed for 1 hour against distilled water under the same conditions as described above and afterwards concentrated *in vacuo* at  $+15^\circ\text{C}$  to about

15 ml. The concentrated solution was dialyzed for 4 hours against the cataphoretic buffer solution under the same conditions as described above. The buffer solution was renewed after 60 and 150 minutes. The contents of the cellophane tube were centrifuged from a precipitate which formed during the dialysis. The centrifugate contained about 2 mg of protein nitrogen per ml. The ionic strength of the buffer was 0.15 (0.05 of acetate buffer + 0.1 of sodium chloride). The centrifuged solution was placed in a Tiselius electrophoresis apparatus of the usual construction with capillary levelling system, and run for 28,800 seconds at 30 mA. After this time the small fraction alone filled the top cathodic cell and was collected separately.

The active fraction with cathodic migration was only slightly yellow in colour. The main part of the colour remained in the stationary fraction. The contents of the top cathodic cell contained about 0.13 mg of protein nitrogen and about 3 units of activity per ml of solution or, calculated per mg of nitrogen, about 20 units, which implies a tenfold additional purification of the factor. It was planned to collect the contents of the top cathodic cell from 15 cataphoretic experiments in order to carry out a new cataphoresis with this material. The series of experiments was made and the collected contents of the top cathodic cell were prepared for a new cataphoresis in the following way. The slightly yellow solution, with a volume of 55 ml, was dialyzed at 0° C for 1 hour against distilled water as described above. A rather heavy precipitate which formed in the cellophane tube during the dialysis was centrifuged off. The centrifugate was still slightly yellow-coloured. The precipitate was dissolved in 10 ml of distilled water by adjusting the hydrogen ion concentration to pH 7.4. The solution was clear and colourless and contained about 85 per cent of the original activity in the cellophane tube. The activity calculated per mg of protein nitrogen was still about 20 units (22). This figure certainly did not represent the maximum activity obtainable. The series of electrodialyses was carried out during the course of about 2 weeks. On the basis of previous experience it was to be suspected that the activity of at least the first samples in the cataphoretic series was partially destroyed during storage in the frozen state. The activity must also have been diminished by the repeated dialysis, which slowly splits the enzyme into two inactive components (ÅGREN 1945 a and b).

Several series of cataphoretic experiments were carried out

in this manner, but the results were not always reproducible. It sometimes happened, especially when the activity of the cataphorized and stored material was low, that the active substance was not precipitated in the subsequent dialysis against distilled water. The solution of the active precipitate gave the usual colour reactions for amino acids.

### Discussion.

From a previous work (LIECK and ÅGREN 1944) it was known that extracts of carp viscera destroyed more thiamin than similarly prepared extracts of viscera from ide. It was therefore planned to purify the thiamin-splitting factor from carp viscera. When a comparison was made of the activities of water extracts of viscera from carp and ide prepared in the same way, they were usually found to contain about 0.6 and 0.15 units of activity respectively, calculated per mg of protein nitrogen. However, it was soon found impossible to apply the previously outlined method of preparation (ÅGREN 1945 a) to such extracts. Several other methods generally used in the purification of protein compounds were unsuccessful when tried on extracts of carp viscera of different dilutions and pH values. At present extracts of ide are preferred for purification of the thiamin-splitting factor.

The biological significance of the thiamin-destroying factor is still unclear. In a previous paper it was suggested that the factor might be engaged in the control of the synaptic transmission of nerves (ÅGREN 1945 b). This hypothesis would seem to presume a wide spread distribution of the factor. At present it has only been found in different tissues of fish. A second possibility must also be considered. In a recent paper BELOFF and STERN (1945) demonstrated that yeast carboxylase of different states and degrees of purity was appreciably inhibited by treatment with extracts containing the thiamin-destroying fish factor. The reaction probably involved a destruction of the cocarboxylase component of the enzyme. The authors did not discuss the physiological significance of their experiments. It is clear, however, that if the results are confirmed with carboxylase preparations from fish, the thiamin-inactivating factor may be involved in the regulation of oxygen consumption in a manner similar to that exhibited by vitamin E, which has an inhibitory effect

on the oxygen consumption of the succinic dehydrogenase system (HOUCHIN 1942). Since it has been demonstrated in the present paper that the thiamin-inactivating fish factor appears to be a colour-free protein, it would be of interest to investigate whether the dialyzable co-factor is identical with glutathione or some other reducing substance (cf. ÅGREN 1945 a and b).

### Summary.

By cataphoretic separation and dialysis the thiamin-destroying fish factor has been further purified about 10 times, in all about 100 times. The activity seems to be associated with a colourless protein.

The author is indebted to the Astra Corporation for a grant which supported the present investigation. He further expresses his thanks to Mr. Eklund for valuable assistance throughout the investigation.

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## The Gastric Lipase in Man.

By

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Received 23 March 1946.

The literature on the gastric lipase has been thoroughly reviewed by OPPENHEIMER (1925, 1936) and since then very few papers on the gastric lipase have appeared. Whereas VOLHARD and his school were inclined to ascribe a considerable importance to the gastric lipase in the digestion of fats in the stomach both in dog and man, it is at present taught that in the adults the gastric lipase is of little or no importance in the digestion of fat. As regards the importance of the gastric lipase in infants the question is less clear. According to OPPENHEIMER the existence of a characteristic gastric lipase has never been proved in the human gastric juice, as it is claimed that in the investigations there has not been paid sufficient attention to regurgitation of pancreatic lipase from duodenum to the ventricle. As late as in 1928 MELLI and RADICI have published results which indicate that the lipase present in gastric juice from normal persons most likely is identical with the pancreatic lipase. The majority of investigations on the gastric lipase are rather ancient and have been carried out mostly with fats which were not well defined.

We have been able to confirm that gastric juice from adults and infants contains a characteristic gastric lipase, which is identical with the lipase present in the gastric mucous membrane. It is shown that as in the case of pancreatic lipase the optimum pH for gastric lipase is very dependent on the triglycerides used as substrate, the optimum pH for the lower triglycerides being about 5.5 and for the higher triglycerides about 7.5. In case of the higher triglycerides  $\text{CaCl}_2$  is able to shift the optimum

pH to the acid side. The human gastric lipase is a very stable enzyme in the acid medium. In vivo only the lower triglycerides seem to be hydrolyzed to an appreciable degree by means of the gastric lipase and apparently there is no basis for assuming that this enzyme plays a greater rôle in the fat digestion in infants than in adults.

### Methods.

*Substrates.* The substrates employed were: tripropionin, tributyrin, trieaproin, tricaprylin, triaprin, trilaurin, tristearin, triolein, cow-butter fat and woman's milk fat.

*Enzyme.* As enzyme preparations were used either glycerol extracts of dried gastric membrane or aspirated fasting gastric juice. Glycerol extracts of the dried mucous membrane were prepared according to directions given in OPPENHEIMER (1925) with slight modifications. Very shortly after death the stomachs of clinically healthy men, killed in accidents, were opened and the mucous membrane was carefully rinsed with water and wiped. The mucous membrane of fundus ventriculi was then scraped off, comminuted and dried with acetone, acetone-ether and ether. The dried powder was extracted with 1/40 n NH<sub>4</sub>OH. The extract was precipitated with acetic acid and the precipitate dissolved in water by addition of small amounts of NaOH to get a clear solution. Glycerol was added and the solution was concentrated in vacuo, 2 mm Hg, to a mixture containing about 50 % of glycerol. In the experiments on the determination of the activity of the extract towards different triglycerides extracts and dilutions 1/50 were used. Gastric lipase in gastric juice from adults was obtained by aspirating the total fasting secretion. In order to get gastric juice from children it was necessary to rinse the stomach with a small amount of water, as generally no fasting secretion could be aspirated without this measure. The fasting secretions in adults were occasionally coloured by bile, whereas the rinse-water from children was always colourless. As will be seen later, there was in no case any reason to assume that these secretions contained other lipases than the characteristic gastric lipase. In order to preserve the lipase in the secretions these were brought to a pH about 5.6 immediately after the withdrawal.

*Determination of the Enzymatic Hydrolysis.* a) *Experiments in Vitro:* The lipolytic activities of solutions containing gastric lipase towards different triglycerides were determined as in our previous papers, SCHÖNHEYDER and VOLQVARTZ (1944, 1945). The amount of acid liberated during the reaction was neutralized by adding dropwise 0.1 n NaOH, keeping pH almost constant, the temperature being 40° C ± 0.5° C. The buffers used were acetic acid-sodium acetate and Michaelis' veronal buffer, both 0.1 n. Regardless of solubility 0.253 millimol triglyceride in 30 ml reaction mixture were used for each experiment. The initial velocity was calculated graphically and given

as number of drops of 0.1 n NaOH per 10 minutes (1 ml = about 45 drops). The accuracy of the determinations of the lipolytic activity was about 5 %.

b) *Experiments in Vivo:* The lipolysis in the stomach was tested in the following way. In the adult the fasting content of the stomach was aspirated and the stomach rinsed twice or more with water until the aspirated water was clear and the stomach emptied completely. Then the following test meal was given. 7.5 g peptone and  $\frac{1}{200}$  mole of triglyceride in 150 ml of water. The meals containing the lower triglycerides and triolein were easily emulsified, whereas emulsions of the higher triglycerides were more difficult to obtain. They were melted, peptone solution added and by careful stirring a fairly good emulsion was produced. The meal was ingested through the stomach tube. 25 minutes after the finished ingestion the total content of the stomach was aspirated through the tube. This experimental period was chosen because the pH of the contents of the stomach from that time generally reached a value at which no significant lipolysis is supposed to take place. Peptone was added as a buffer substance. The small amount of triglyceride was chosen on account of the difficulty of obtaining these substances in pure state. In children the test meal was given after previous washing of the stomach with a few ml of water, as the stomach generally was empty. The test meal for children contained the same amount of triglyceride as in adults but only 1.5 g peptone in 150 ml.

On the material recovered from the stomach determinations of total and free acids were carried out. Control experiments with some of the subjects examined showed that their fasting secretions contained no free fatty acids. In the case of tributyrin meals the amount of free acid was determined by extracting 5 ml of the stomach content with 4 times 10 ml petrolether. The aqueous phase contained all the free acid and no triglyceride, whereas the petrolether contained all the triglyceride. In case of tricaprin and tricaprylin the free acid was somewhat soluble in petrolether and therefore the meal was neutralized before extraction. The aqueous phase contained all the free acid as salt. The amounts of free butyric, caproic and caprylic acid were determined by steam distillation of the acid into a receiver containing a known amount of hydrochloric acid. CO<sub>2</sub>-free air was bubbled through the distillate for 15 minutes before titration with  $\frac{1}{10}$ — $\frac{1}{50}$  n NaOH. The total amount of these acids were determined by saponification of suitable amounts of the stomach content and subsequent distillation of the fatty acids. In the case of the higher triglycerides the triglyceride and the free acid were both extracted from the stomach content, made strongly acid, by means of petrolether. The aqueous phase was discarded. The petrolether phase was washed with water and dried with sodium sulphate. The petrolether was then distilled off and the fatty residue dried to constant weight. The residue was then dissolved in alcohol-ether mixture and the free acid titrated with  $\frac{1}{10}$ — $\frac{1}{50}$  n NaOH. The total amount of fatty acid was either calculated from the weight of the residue or determined by saponification of the residue.

Both in the case of the lower and the higher triglycerides the technique has been checked on known solutions of free acids with and without triglycerides.

In a few experiments the degree of hydrolysis of cow-butter fat and woman's milk fat was determined. The woman's milk fat was prepared by extracting the upper fatty layer of the centrifuged milk with petrolether. The petrolether phase was washed with water and dried with sodium sulphate and the petrolether was then distilled off.<sup>1</sup> The test meals contained fatty substance corresponding to about  $\frac{1}{200}$  equivalents of fatty acids. The degree of hydrolysis was in these cases calculated as the difference between the percentage of equivalents of free acids in the aspirated meal (both in the petrolether extract and the aqueous phase) and in the fat before ingestion.

### Experimental.

A. *The Optimum pH of Human Gastric Lipase with and without Addition of CaCl<sub>2</sub> to the Enzyme-Substrate System.* DAVIDSOHN (1912) and HAUROWITZ and PETROU (1925) and GYOTOKU (1928 a) have determined the optimum pH of the human gastric lipase in the gastric secretions using the stalagmometric method and tributyrin as a substrate. They found pH optima about 5 to 6. SCHÖNHEYDER and VOLQVARTZ (1945) have in experiments with pancreatic lipase found, that with increasing number of carbon atoms in the fatty acids of the triglycerides the optimum pH is displaced towards higher values, and therefore similar experiments might be of interest in connection with the gastric lipase. Our experiments with glycerol extracts of dried gastric mucous membrane are carried out with and without addition of calcium chloride (50 mg per 30 ml reaction mixture). The results of the experiments with tripalmitin, tributyrin, tricaprin, tri-caproin, trilaurin and tristearin are given Fig. 1. The ordinates give the initial velocities in per cent of the maximum velocity in the system containing CaCl<sub>2</sub>. The maximum velocity was calculated graphically. In the experiments without addition of calcium chloride it is seen that the optimum pH for the lower triglycerides (tripalmitin, tributyrin, and tricaprin) is found between 5.5 and 5.8. By increasing number of carbon atoms in the fatty acids there is an evident displacement of the optimum pH to the alkaline side, the optimum pH for tricaprin, trilaurin and tristearin being 7.2, 7.3 and 7.9 respectively.

<sup>1</sup> The authors are indebted to the Chief of the Lying-in Hospital for Jutland, AXEL OLSEN, M. D., for supplying the milk samples.

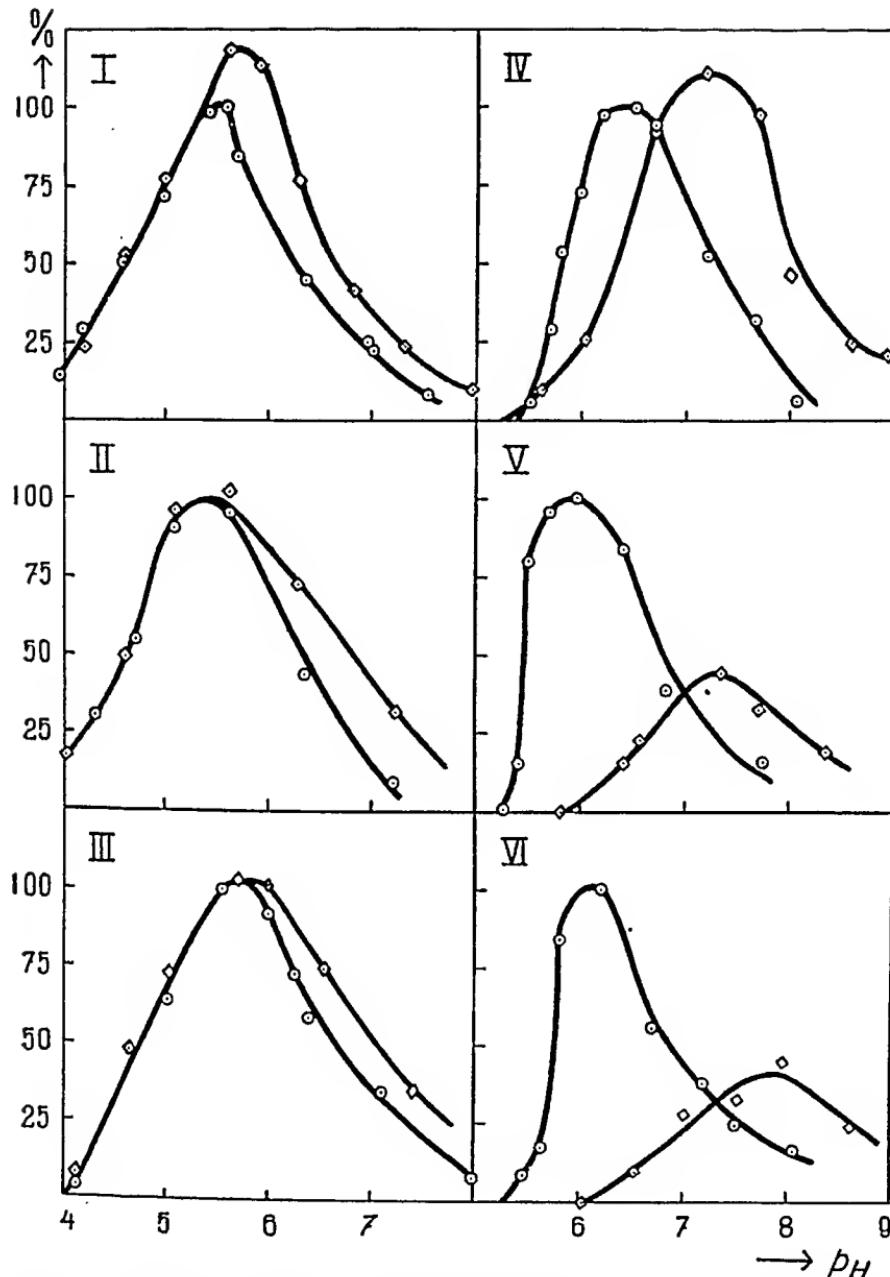


Fig. 1. Velocities of hydrolysis of triglycerides as functions of pH.  $\diamond$  represents experiments without  $\text{CaCl}_2$ ;  $\circ$  addition of  $\text{CaCl}_2$ . (I: tripeptienin, II: tributyrin, III: tricaproin, IV: tricaprin, V: trilaurin, VI: tristearin). Abscissa: pH. Ordinate: Velocities in per cent of the maximum velocity in systems containing  $\text{CaCl}_2$ .

The question concerning the influence of the calcium ion on the activity of gastric lipase has apparently not been investigated previously. Our experiments show that in all the lower triglycerides investigated the addition of calcium chloride to the enzyme-substrate system is found to inhibit the activity of the enzyme at pH-values greater than optimum pH. In the case of trilaurin and tristearin where the free fatty acids are insoluble in water there is a definite activation of the process at pH lower than 7, and the optimum pH for the process is shifted 1.5 to 2 pH units to the acid side. In the case of these triglycerides a definite inhibition is stated at pH higher than 7. The triglyceride tricaprin takes up an intermediate position. As triolein was only split in a very low degree by our glycerol extracts both with and without addition of calcium chloride the experiments on this substrate are not included in Fig. 1.

It should be mentioned that the pH-activity curve for tributyrin was determined in one case with a fasting secretion from an adult person. In this experiment a curve was found with quite the same shape and pH optimum as the curves in Fig. 1 (II). Apparently the enzyme in fasting secretion is therefore identical with the enzyme prepared by extraction of the dried mucous membrane.

B. *The Relative Activity of Gastric Lipase towards Different Triglycerides.* The experiments in Fig. 1 have all been carried out by means of extracts of the same gastric mucous membrane. The activities of the extracts used towards the different triglycerides, calculated for the same amount of enzyme and number of equivalents of substrate, were very different, which appears from Table I. In this table are given the relative initial velocities ( $K_{rel}$ ) at the respective pH-optima.  $K_{rel}$  is given for systems with and without  $\text{CaCl}_2$ , and the velocities are related to an arbitrary value of 100 for tributyrin. It is seen that the relative activities towards the solid triglycerides are very small.

The absolute value of activity towards tributyrin of 1 ml fasting secretion from adults is about 0.45 ml of  $1/10$  n NaOH per 10 minutes in the reaction mixture previously described.

C. *The Stability of Gastric Lipase at Different pH-values.* Investigations on the stability of the lipase present in the gastric secretion of man at different pH-values are not known. From our experiments (see Fig. 2) it appears that the enzyme is very stable in the pH range from 3 to 7 and that in the range between 2 and 7.5 the half decomposition time for the enzyme is more

Table I.

*Relative Initial Velocity of Hydrolysis of Triglycerides by Human Gastric Lipase.*

Triglyceride	$K_{rel.}$ without $\text{CaCl}_2$	$K_{rol.}$ with $\text{CaCl}_2$
Tripropionin . . . . .	27	23
Tributyryl . . . . .	100	100
Tricaproin . . . . .	39	39
Tricaprin . . . . .	13	12
Trilaurin . . . . .	2	5
Tristearin . . . . .	0.8	2
Triolein . . . . .	negligible	negligible

than 7 hours, the fasting secretions being kept at 40° C. The different hydrogen ion concentrations were obtained by adding HCl and NaOH to the secretions. The inactivation follows the equation of the monomolecular reaction, thus the half decomposition time can be determined fairly accurately. It appears that the gastric lipase in man is a very stable enzyme, and there is hardly any doubt that considerable amounts of gastric lipase get into the intestine in non-destroyed condition, where it may support the pancreatic lipase in the digestion of fatty substances. Also Gyotoku (1928 b) is of the opinion, that gastric lipase in man is an enzyme that is remarkably stable in comparison to other lipases, but his investigations are carried out on extracts of gastric mucous membrane, and the pH values at which his examination of the stability were carried out are not given.

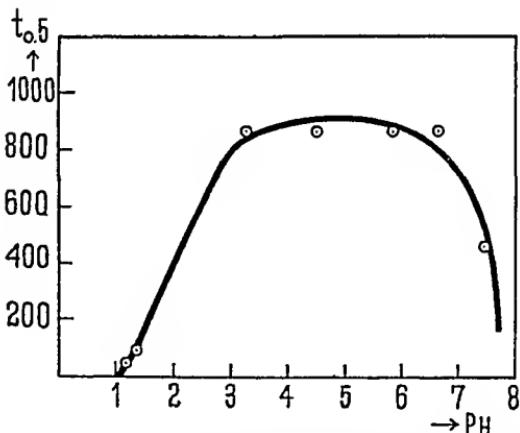


Fig. 2. Determination of the stability of human gastric lipase in gastric juice at different pH at 40° C. The ordinates represent the half decomposition time in minutes.

D. *Experiments on the Lipolysis in the Stomach.* The total gastric digestion of fat in human subjects can not be determined, as such determinations necessitate a duodenostomy or duodenal fistula from which the gastric discharge can be collected as it is being evacuated through the pylorus. The only satisfactory alternative is to withdraw the gastric content by means of a tube. This only yields information on the state of digestion at the time the samples were obtained, but when the withdrawal takes place at the same time after the intake of the meal it is possible to get a relative measure of the digestion of the different triglycerides in the stomach, and one can state whether there is any quantitative difference between the digestion of fat in adults and in children. The technique has been reported above, and 6 adults and 6 children were used as subjects for these experiments. The adults were 3 males and 3 women, clinically normal, only No. 6 was suffering from achylia. The 6 children were younger than 9 months and they were patients in the Department of Pediatrics, Aarhus Kommunehospital.<sup>1</sup> The experiments took place at the time at which the children were convalescent after diseases, which could not be supposed to influence the stomach function. The results obtained from 18 test meals in children and 20 test meals in adults are summarized in Table II. For each aspirated meal the pH and the percentage of fatty acid liberated in relation to total amount of fatty acid in the test meal are given. A correction for free acid in the triglyceride ingested has of course been introduced. The degree of hydrolysis relates only to the state of lipolysis of the triglyceride remaining in the stomach. That which had been evacuated into the intestine was perhaps less hydrolyzed. Although the test meals given to children contained considerably less peptone than those given to adults the pH values in the recovered material were much higher than those found in adults, No. 6 excepted. Nevertheless there is no appreciable difference between the degree of hydrolysis in children and adults. In each group the degree of hydrolysis of the low-molecular triglycerides is large, whereas in the higher triglycerides there is a tendency to a higher degree of hydrolysis in the case of children. The absolute percentage of acid liberated is, however, also in children so slight that it is of no practical importance.

Whereas the order of magnitude of the degrees of hydrolysis

<sup>1</sup> The authors are indebted to Professor BENT ANDERSEN, M. D., for permission to make these investigations in his department.

**Table II.**  
*The Hydrolysis of Pure Triglycerides in the Ventricle of Adults and Infants.*

found for one and the same triglyceride with different subjects was fairly uniform the absolute amounts of fatty substance varied greatly from one individual to another. These values are not included in Table II, but it should be mentioned that we generally aspirated a much greater percentage of the lower triglycerides and triolein (10 to 55 %) than in the case of the higher saturated triglycerides (3 to 15 %).

The experiments in Table II were supplemented with a few experiments on the digestion of cow-butter fat and woman's milk fat in the stomach of children. The test meals contained the same number of equivalents as used above (3/200 eqv.), calculated from the saponification of these fats before ingestion. Fine emulsions were prepared in 150 ml of water containing 1 % peptone. Details concerning the procedure have been given previously. The results of these experiments are given in Table III.

Table III.  
The Hydrolysis of Cow's Milk Fat and Woman's Milk Fat in the Ventricle of Infants.

	Child No. 7 aged 7 months		Child No. 8 aged 6 months	
	Cow's milk fat	Woman's milk fat	Cow's milk fat	Woman's milk fat
pH in recov. meal . . .	4.77	5.43	3.77	4.38
Fat recovered . . . .	46 %	56 %	58 %	23 %
Acid liberated . . . .	6.87 %	4.1 %	6.71 %	4.87 %

All meals have been ingested after it had been ascertained that the stomach was empty. In 19 fastings secretions from adults and in 8 samples of fasting wash-water from children the lipolytic activities towards tributyrin were determined at pH 5.5 and 8.0. The optima pH for gastric lipase and pancreatic lipase respectively materially greater than 1, generally about 20, and in each case the activity at pH = 8 was negligible. This finding agrees best with the assumption that the lipolysis which takes place in the stomach is caused by a characteristic gastric lipase. Even in fasting secretions from adults containing bile it was not possible to prove the existence of a pancreatic lipase. In no aspirated

material was there any sign of regurgitation from the intestine. The only fatty substances which seem to be digested in the stomach to a considerable degree were the lower triglycerides. From our results with test meals the conclusion may be drawn that the gastric lipase hardly asserts itself to a greater extent in infants than in adults (cf. the experiment with tricaproin). It had been stated (PESTHY 1906), that in patients suffering from achylia the gastric lipase is not missing, which appears, too, in our experiment with adult No. 6.

Our experiments on the digestion of milk fat in the ventricle of 2 infants show that there is only a slight difference between the splitting of the two kinds of fat during the experimental period (25 min.). However, the degree of hydrolysis is somewhat smaller in the case of woman's milk fat than for the cow's milk fat. This was to be expected as it is well known that woman's milk fat contains fewer of the low-molecular triglycerides than cow's milk, (see among others HILDITCH and MEARA, 1944).

We think it is not without interest that the splitting of cow's milk in the stomach of infants by means of gastric lipase is found to be a little greater than in the case of woman's milk.

### Summary.

A reexamination has been carried out of the problems concerned with the gastric lipase in man, mainly with pure triglycerides as substrates. Both *in vitro* and *in vivo* experiments were undertaken.

1. The optimum pH for gastric lipase towards tripropionin, tributyrin and tricaproin in systems without the addition of  $\text{CaCl}_2$  is found to be between 5.5 and 5.8. With an increasing number of C-atoms in the fatty acids there is an evident displacement of the optimum pH to the alkaline side, the optimum pH for tricaprin, trilaurin and tristearin being 7.2, 7.3 and 7.9 respectively.

2. Addition of  $\text{CaCl}_2$  to the enzyme-substrate systems causes definite activation of the hydrolysis of trilaurin and tristearin at pH lower than 7, and the optimum pH is shifted 1.5 to 2 pH units to the acid side.

3. The relative activities of gastric lipase towards different triglycerides have been computed. Tributyrin is split with the

greatest initial velocity, whereas the relative activities towards the solid triglycerides are very small.

4. Experiments on the stability of gastric lipase at 40° C at different pH values show that gastric lipase is a very stable enzyme in the acid medium.

5. The lipolysis stated in our experiments *in vivo* is according to our opinion due to a specific gastric lipase and not to a regurgitated pancreatic lipase. Experiments *in vivo* with test meals containing pure triglycerides show that only the lower triglycerides are split to an appreciable extent during the experimental period of 25 min. Under similar experimental conditions there seems to be hardly any difference between the degrees of hydrolysis of fatty substances in the stomach by means of the gastric lipase in adults and in children. Under similar experimental conditions in children there is a tendency to a higher splitting in the stomach of cow-butter fat than of woman's milk fat when these fats are given in test meals.

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## The Effect of Piperidine and Allied Substances on Mammalian Skeletal Muscle.

By

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Received 30 March 1946.

By means of a special technique, now generally known as "close arterial injection", BROWN, DALE and FELDBERG (1936) showed that acetylcholine in minute doses produces a rapid and vigorous contraction of mammalian striated muscle. The effect belongs to the kind of actions of acetylcholine which DALE (1914) has termed "nicotine-like". It was later demonstrated that various other choline esters produce a similar effect and so does nicotine itself (BACQ and BROWN, 1937).

It has recently been shown at this laboratory (EULER, 1944, 1945 a) that piperidine is a normal constituent of human and animal urine, being excreted — in man — in amounts of 5—20 mg daily. Since piperidine is known to exert a variety of pharmacological actions, typical of nicotine, it seemed of interest to study its effect on the mammalian striated muscle, the more so after it has been shown that piperidine output in urine may be considerably increased during muscular work (EULER, 1945 b).

Apart from piperidine, a number of other hydrated pyridine derivatives, more or less closely related to piperidine, were studied as to their effect on muscle.

### Technique.

Cats, weighing between 2.0 and 3.5 kg, were decerebrated under ether. The tibialis anterior muscle was prepared according to BROWN (1938). The animal was kept in a large, heated metal box serving as

thermostat, moist chamber and electrical screen. The muscle movement was transmitted by means of a string to a BROWN-SCHUSTER spring myograph. The isometric contraction was recorded mechanically on a smoked drum, as well as optically via a piezo-electric crystal with an amplifier-oscillograph system, synchronously with the action potentials. The temperature in the chamber was as a rule kept between 33° and 37° C.

The muscle was stimulated indirectly with condenser shocks, using silver electrodes, placed on the sciatic nerve in the thigh.

The stimulation electrodes for the denervated muscle were placed one at the tendon, and the other in the proximal part of the muscle.

The stimulating shocks were obtained from a neon lamp stimulator and their strength kept but slightly above that giving a maximal single twitch. The muscle was stimulated at regular intervals (approx. 5 sec.) and, at the injection, one impulse was omitted *ad modum* BROWN.

The injections lasted 1—2 sec. and the injected volumes were 0.25 ml.

The action currents were lead off from the tips of two enamelled steel electrodes, attached to each other 1—2 mm apart. After insertion in the muscle they were fixed by means of short flexible wires so as to avoid undue displacement during the contraction. Registration with two differentially coupled amplifiers ("Triplex", System ELMQVIST, Sweden).

The condensor-coupled amplifiers had a relatively large time constant (approx. 2 sec.).

### Solutions.

The temperature of the solutions at the time of injection was 37° C. All stock solutions kept at + 4° C.

Loeke's or Tyrode's solutions, mammalian Ringer's solution, plasma or whole blood were used as vehicles for the examined substances.

*Piperidine*, as piperidinum purissimum, neutralized with HCl. Approximately blood-isotonic stock solution (0.150 molar).

This solution was diluted with Loeke's or Tyrode's solution, whole blood, etc., to the desired strength.

The stock solution was found to cause rapid haemolysis of cat blood corpuscles. However, when made 0.155 molar with regard to NaCl, there was no haemolysis. This latter solution was compared with the original stock solution, both at a dilution of 1 : 5 with Loeke's solution. They gave a similar effect on the muscle as regards the size of the elicited contraction.

In order to exclude calcium shortage, the stock solution was made 0.04 % with regard to Ca Cl<sub>2</sub> in one experiment. This did not noticeably alter its effect on the muscle when used in a dilution 1 : 5 in Loeke's solution.

*Acetylcholine*. Stock solution with 1 mg acetylcholine/ml of dist. water, acidified with HCl to pH 4 and diluted with Loeke's, Tyrode's solution etc., immediately before the beginning of the experiments.

*Nicotine*, as acid l-nicotine-d-tartrate. Stock solution with 0.2 % nicotine in Loeke's solution.

*Coniine*, as hydrochloride. Stock solution with 2 % coniine in dist. water.

*Spartein*, as sulphate. 0.150 molar stock solution (in dist. water).

*Arecoline*, as hydrobromide. 0.150 molar stock solution (in dist. water).

The stock-solutions were always diluted in the same kind of physiological salt solution as in the "blind-tests" of same experiments.

A small and constant dose of heparin in order to prevent clotting was regularly added to the injected solutions.

## Results.

### 1. "Injection effect".

At an early stage in the present investigation it was noticed that sometimes the mere injection of the salt solution, used as a vehicle for the substances to be tested, caused a marked and even strong contraction.

This action of plasma, Locke's solution or other physiological solutions, will be termed "injection effect".

In some preparations the "injection effect" could be demonstrated at the very first injection. In other cases, however, this effect occurred only later on, and often became increasingly marked during the course of the experiment. This latter type of "injection effect" appeared, more or less pronounced, in most of the experiments.

Fig. 1 A illustrates a case where the very first injection (Locke's solution) released a contraction.

The subsequent injection of 2.5  $\mu$ g acetylcholine in "Locke" gave, remarkably enough, a smaller contraction than with "Locke" only. After this, when "Locke" and acetylcholine (in "Locke") were given alternately, the latter in gradually increasing dosages and with successively augmented effects, the former showed a fairly constant action.

In other preparations, however, the initial injections gave no effect. (Cp. Figs 1 B and 1 C.)

Fig. 3 B shows action potentials during an "injection effect" (Tyrode's solution), released by the very first injection made in the preparation concerned. The electrical activity is shorter than at an acetylcholine injection. Still the action is of the type of a short tetanus as shown also by the mechanogram which sometimes exceeded in height the maximal single twitch.

The mechanogram may return to the base-line, after injection of the salt solution only, more quickly than after acetylcholine (Fig. 1 A).

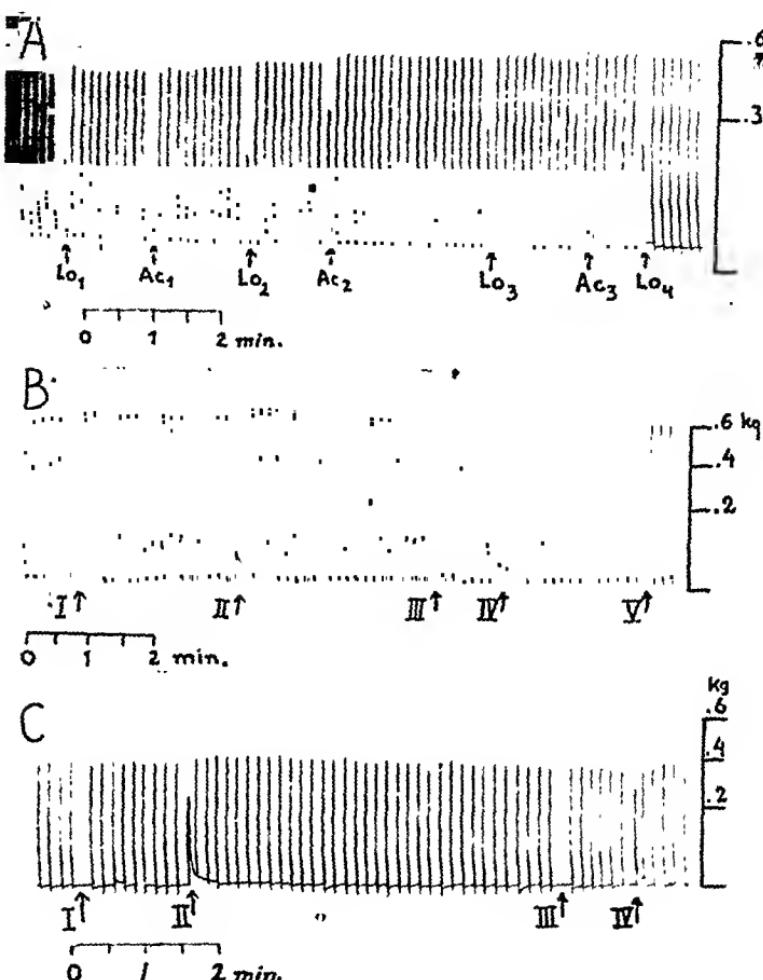


Fig. 1.

A. Cat, 2.4 kg. Tib. ant. Max. indir. stimulation. Close art. injection. At  $Lo_1$ — $Lo_4$ : injection each time of 0.25 ml of Locke's solution. At  $Ac_1$ : 2.5  $\mu$ g of acetylcholine in 0.25 ml of "Locke". At  $Ac_2$ , in the same way 5  $\mu$ g of acetylcholine, and at  $Ac_3$ , 10  $\mu$ g of acetylcholine. The height of the contractions due to the "Locke"-injections (s. c. injection effect) approximately the same on all four occasions. Proportional to the dosage, acetylcholine gives increasing contraction heights.

B. Cat, 2.4 kg. Tib. ant. Max. indir. stimulation. Close art. injection. At I: Locke's solution. No effect. II: 600  $\mu$ g of piperidine in "Locke". Contraction. III: Only "Locke". No effect. IV: 2.5  $\mu$ g of acetylcholine in "Locke". Contraction. V: "Locke". Indicated contraction.

After piperidine, slight potentiation of the single twitches; practically none after acetylcholine.

C. Cat, 2.7 kg. Tib. ant. Max. indir. stimulation. Close art. injection. Analogous to fig. 1 B I, II, III, IV respectively, the same as in Fig. 1 B. Note: duration of piperidine contraction and potentiation of subsequent twitches.

The tendency towards an "injection effect" appearing later on in the experiment is, apparently, to some extent due to the substances used in previous injections.

Piperidine was found to increase the tendency in this respect (cp. Fig. 6 A).

In one case, where the nerve muscle transmission had been completely blocked by spartein, contraction could still be released by the injection of plasma or whole blood. In one experiment the injection of only 0.05 ml elicited a small but distinct contraction.

One preparation which did not contract from the injection of mammalian Ringer's solution of 37° C, remained unaffected even by the injection of "Ringer" of 0° C and other temperatures in between.

## 2. Piperidine.

The piperidine effect was studied in 25 animals. In each experiment repeated injections were made under varying conditions.

Piperidine injections released a short tetanus, as already mentioned, lasting between 0.5 and 2 sec. Cp. Fig. 2. The doses equalled between 50 and 1,000  $\mu$ g. 50  $\mu$ g sometimes gave a small, sometimes no contraction in the normal muscle.

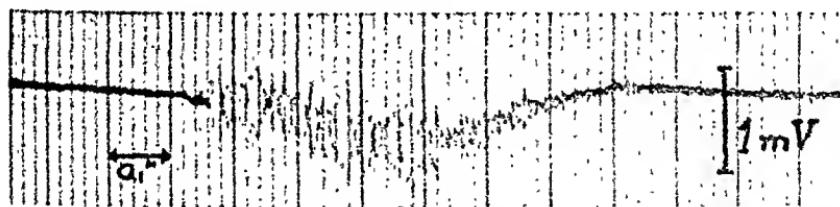


Fig. 2. Cat. Tib. ant. Close art. injection of 250  $\mu$ g piperidine. Asynchronous tetanus for some 0.5 seconds.

As compared to acetylcholine, the piperidine myogram rises more gradually. Fig. 3 A shows the myograms of the contractions II and IV in Fig. 1 B, as registered with the piezo-electric device. In other cases, the difference was still greater.

Even the relaxation is slower. Apart from Fig. 3 A, this is noticeable in Fig. 1 C (II), in spite of the slow rotation of the drum.

Fig. 2 shows an irregular tetanus, released by 250  $\mu$ g of piperidine in Ringer's solution. Pure mammalian Ringer's solution gave no contraction and no action potentials in this preparation.

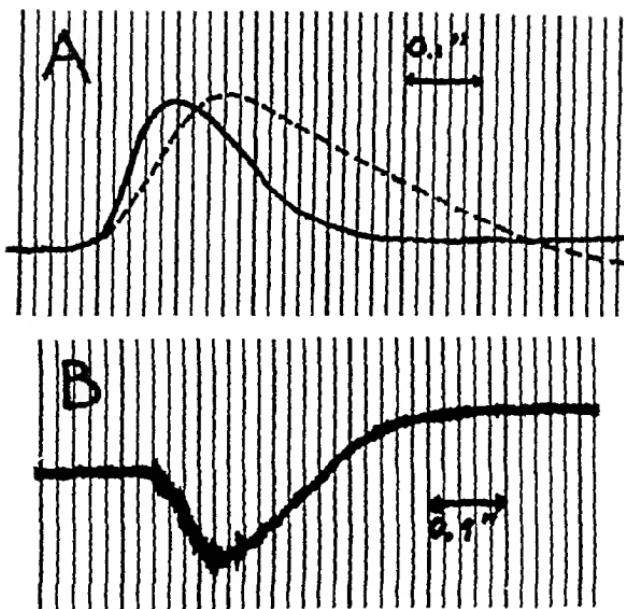


Fig. 3.

A. Reproduction of mechanogram (piezo-electric registration) from acetylcholine (continuous curve) and piperidine contraction (dashed curve), II and IV in Fig. 1 B. B. Slower rise and fall, respectively, of the piperidine curve.

B. Cat, 2.6 kg. Tib. ant. Close art. injection of Tyrode's solution. Action potentials: the activity lasts for a good 0.1 second.

In Fig. 4, the activity in what appears to be a single muscle element is registered. This series of discharges started 2.3 sec. after the beginning of the initial asynchronous tetanus (= contraction II in Fig. 1 C). Fig. 5 depicts graphically the relationship between the interval of the single spikes and the time at which the activity in question first manifested itself. Similar curves for acetylcholine are given by BROWN (1937 a).

Successive injections of 50, 125 and 250  $\mu\text{g}$  of piperidine gave contraction heights with an approximate ratio of 1 : 2 : 3.

The effect of piperidine on the subsequent single twitches varied. Figs. 1 B and 1 C show a slight, transient post-piperidine



Fig. 4. Cat, 2.7 kg. Tib. ant. Close art. injection of 600  $\mu\text{g}$  of piperidine. Action potentials registered. 2–3 seconds after the start of the piperidine effect, the reproduced series of spikes set in. The corresponding mechanogram is seen at "II" in Fig. 1 C. 1 mV = 15 mm.

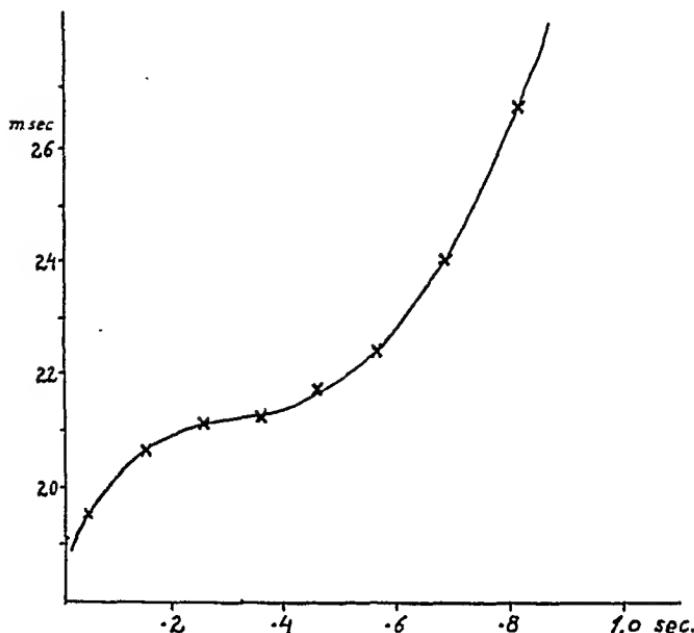


Fig. 5. Graphic analysis of Fig. 4. Abscissa: time from beginning of the serial discharge concerned. Ordinate: intervals between the single spikes (in milliseconds). Each point of the curve represents the arithmetic medium of five intervals.

potentiation, whereas Figs. 6 A and 6 B disclose an inhibition which, in the latter case, reaches maximum after about 20 sec., and has entirely disappeared after 2 or 3 minutes.

The potentiation is probably related to the post-tetanic potentiation studied by BROWN and EULER (1938), since the piperidine contraction is, in fact, a short tetanus. In some experiments, a distinct potentiation was observed also after acetylcholine injection.

The inhibition, on the other hand, is most likely a manifestation of the curare-like action exerted by nicotine and nicotine-like substances, including also acetylcholine (cp. Figs. 6 C and 7 D). The curare-like action of piperidine was observed by CUSHNY (1896) and others.

After denervation the muscle becomes very sensitive to piperidine. Fig. 7 A shows the effect of 50  $\mu$ g piperidine in a cat weighing 2.8 kg in which the sciatic nerve had been sectioned one month earlier. The same experiment demonstrates (at "I" in Fig. 6 C) the effect of 0.25  $\mu$ g acetylcholine, and at II, the effect of 600  $\mu$ g of piperidine. The acetylcholine gave the characteristic

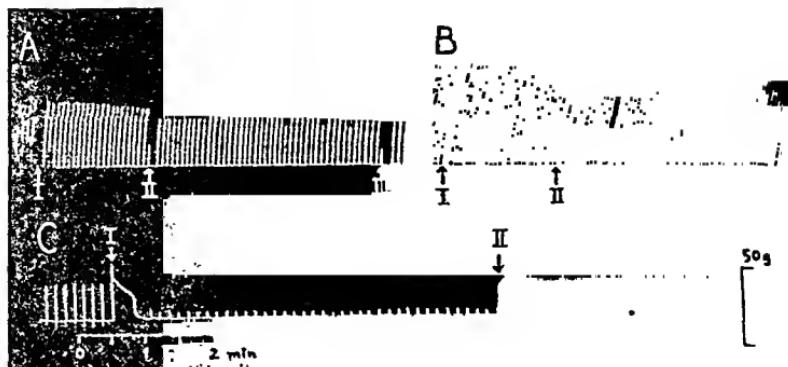


Fig. 6

A. Cat, 2.3 kg. Tib. ant. Max. indir. stimulation about 5 sec. apart. Close art. injection I: injection of mammalian Ringer's solution. No effect. II: 100 µg of piperidine. Contraction and subsequent inhibition of the single contractions. III: Again "Ringer". This time contraction, i.e. "injection effect". The contraction level slightly lower than that of piperidine. No action on the subsequent single twitches.

B. Cat, 2.0 kg. Tib. ant. Max. indir. stimulation about 5 sec. apart. Close art. injection. I: injection of 5 µg acetylcholine. Contraction nearly as high as the single twitch. II: 250 µg of piperidine. The contraction now approximately half as big as at A. Reversible inhibition of subsequent twitches. Inhibition was maximal approximately 20 sec. after the injection and completely disappeared after 2 or 3 minutes. Possibly the electrically induced twitches are very short tetani in this experiment (ep. Figs. 7 B and 7 D).

C. Cat, 2.8 kg. Tib. ant. Denervated one month previously. Direct stimulation of muscle. Close art. injection. I: 0.25 µg of acetylcholino. "Biphasic" contraction curve and subsequent inhibition of the single twitches. At II, injection of 600 µg of piperidine. Contracture, which did not relax within 3 minutes.

"disphasic" curve (BROWN, 1937), and a partial inhibition of subsequent single twitches. Piperidine caused a contracture which did not relax during the following 3 minutes.

### 3. Nicotine, coniine, spartein and arecoline.

*Nicotine.* (5 cats.) A dose of 15 µg of nicotine elicited a distinct contraction and slight inhibition of subsequent single twitches. Fig. 7 C shows the effect of 150 µg of nicotine: the contraction level is higher than that of the single twitches. After this dose indirect stimulation gave no contraction. Ringer's solution just before gave no "injection effect".

*Coniine.* (5 cats.) Fig. 7 D illustrates an experiment where a previous injection of Tyrode's solution gave no "injection effect". 250 µg of coniine, however, gave contraction and subsequent inhibition of the indirectly induced contractions. Each of these contractions corresponds to a short tetanus.

*Spartein.* (4 cats.) This compound elicited but little or no con-

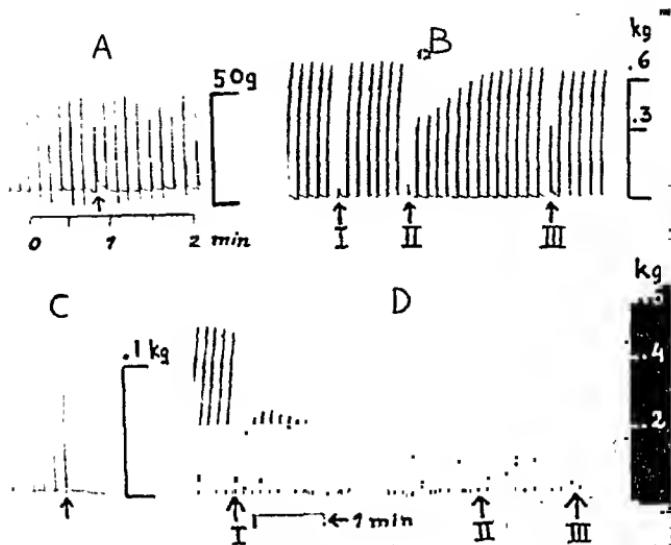


Fig. 7.

A. Cat, 2.8 kg. Tib. ant. denervated one month previously. Direct stimulation of muscle.

At "↑" 50 µg of piperidine were given: distinct contraction.

B. Cat, 2.2 kg. Tib. ant. Supramax. indir. stimulation (giving short tetani) about 5 sec. apart. Close art. injection. I: Tyrode's solution. Small contraction ("injection effect"). II: 140 µg of spartein. The contraction slightly bigger than after "Tyrode" only. Reversible inhibition of subsequent contractions. III: 10 µg of acetylcholine. Contraction bigger than at I and II.

C. Cat, 2.5 kg. Tib. ant. Max. indir. stimulation. Close art. injection. At "↑" 150 µg of nicotine were injected. Contraction. Subsequent nerve stimulation no longer caused any effect on the muscle.

D. Cat, 2.7 kg. Tib. ant. Supramax. indir. stimul., giving short tetani at equal intervals. Close art. injection. I: 150 µg of coniine. Contraction and inhibition of subsequent contractions. II: 10 µg of acetylcholine. III: 250 µg of acetylcholine. After III, stimulations of the nerve give practically no contractions.

traction effect. In one test, where 2.5 µg of acetylcholine gave a distinct contraction, 0.9 mg of spartein gave neither contraction nor any effect on subsequent single twitches. In another preparation, 0.25 mg spartein gave some contraction (hard to interpret, however, owing to "injection effect"), and then a total neuromuscular blockage. In other experiments, again, a reversible inhibition of subsequent contractions was observed (Fig. 7 B).

*Arecoline.* (3 cats.) Doses of 0.6, 1.2 and 6.0 mg respectively, produced no contraction in a preparation with normal sensitivity to acetylcholine and piperidine. Subsequent single twitches were inhibited to a varying degree, the inhibition being roughly proportional to the dosage. Also in other experiments arecoline was inactive with regard to the contraction effect.

### Discussion.

The fact that the mere injection of a physiological solution (Locke's solution, whole blood, etc.,) may elicit a muscle contraction is of some interest, primarily perhaps, as a source of error. It should be emphasized that large amounts of fluid are not required for the producing of this effect which could be observed after as small a volume as 0.05 ml, and without using a particularly high injection velocity. After total neuromuscular blocking (by means of spartein), small contractions could still be released by plasma or whole blood. This suggests that the point of attack of the "injection effect" may be further peripheral than the motor neuron.

The closer nature of this effect (mechanically or chemically — variations in pH? — elicited stimulation) was not investigated.

The demonstration of the capacity of piperidine to induce a tetanus has lead to the ascertainment of still another body-specific substance capable of releasing a contraction in mammalian skeletal muscle.

Analogously to acetylcholine and nicotine (LANGLEY, 1913/14, BUCHTAL *et al.* 1942, and others), the point of attack of piperidine in the muscle is probably the end-plate.

As regards the observations made by BUCHTAL *et al.* (1944) concerning a contraction released by adenosine triphosphoric acid and related compounds, this effect is apparently connected to structural changes of the myosine molecule.

Compared with acetylcholine, an about 200 times larger dose of piperidine was needed in order to cause the same contraction height. However, such a comparison seems somewhat objectionable since piperidine gives a slower effect regarding the period of contraction as well as the relaxation time.

The capacity of piperidine to produce contractions in the isolated rectus abdominis of the frog (EULER and DOMEIJ, 1945) is not necessarily analogous to the effect described in the present paper. Thus, they behave differently with regard to acetylcholine (cp. BROWN, 1937). Another apparent discrepancy between amphibian and mammalian muscle is to be met with in the present paper: arecoline fails to cause contraction, in spite of the fact that it has been found capable of this effect in the muscle of frog.

The action of piperidine on various biological objects has always been found to be weaker, weight for weight, than nicotine. Apparently this also applies to the capacity of producing a contraction effect in mammalian skeletal muscle. The relation between nicotine and piperidine with regard to the effect on the blood pressure equals 20 : 1 (DIXON, 1924), on the reetus abdominis muscle of the frog 200: 1 (EULER and DOMEIJ, 1945). From the present experiments, the ratio may be given as approximately 10: 1.

Coniine causes contraction with inhibition of the subsequent contractions whereas spartein gave slight or no contraction in the doses employed. However, there was at times a distinct, sometimes reversible, inhibition of the subsequent twitches with spartein. This is apparently a curare-like effect, as a contraction could still be elicited by direct stimulation (*i. e.* "injection effect" still demonstrable).

I am greatly indebted to Professor U. S. v. EULER for suggesting the problem and for his inspiring interest in the progress of the work.

### Summary.

1. Piperidine in doses of 50  $\mu\text{g}$  or more produces a short tetanus in the tibialis anterior of the cat, when applied *ad modum* BROWN (1938), *i. e.*, "close arterial injection".
2. The effect of subsequent maximal single twitches is either a transient potentiation or else inhibition (the latter sometimes reversible).
3. The myogram of piperidine has a slower rise and fall than the corresponding acetylcholine curve.
4. Denervated muscle is highly sensitive to piperidine. It causes contraction and, in larger doses, contracture.
5. Injection of Locke's solution, plasma, or some other physiological solution may sometimes produce a short tetanus ("Injection effect").
6. Nicotine and coniine give contraction and subsequent inhibition of indirectly induced contractions.
7. Spartein and arecoline give slight or no contraction, but distinct inhibition of indirectly induced contractions.

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## Effect of Minute Amounts of Barium on Cardiac Muscle.

By

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In the course of investigations on striated skeletal muscle (BUCHTHAL et al. 1944), we have examined the effect of adenosine triphosphate on small bundles of cardiac muscle fibres without automatic activity. Former investigations have established that adenosine triphosphate in moderate concentrations has no effect on normal cardiac muscle, while contraction tension is claimed to increase in "hypodynamic" preparations (LINDNER and RIGLER 1931). Large amounts are found to release block and other disturbances in the conducting system (GILLESPIE 1934).

In our first experiments adenosine triphosphate (ATP) applied to small bundles of cardiac muscle had a striking effect, releasing automatic activity of several minutes duration. This effect, however, could not be reproduced regularly and it was soon realized that it was due to an accidental contamination by small amounts of Ba salts of the ATP solution used in the first experiments. This made us to investigate the effect of Ba salts in different concentrations.

Apart from ATP other phosphorus compounds have been examined with regard to their action on cardiac muscle.

### Method.

The experiments were performed on small approximately parallel threaded muscle bundles 0.1—0.3 mm thick and 1.5—2 mm long, from the cardiac ventricle (*Rana esculenta*), prepared in an ice-cooled Ringer solution of pH 7.3. The Ringer solution contained 0.67 g NaCl, 0.02 g KCl, 0.04 g CaCl<sub>2</sub>, 6 H<sub>2</sub>O and 0.02 g glucose in 100 ml. Oxygen content

<sup>1</sup> Working with a grant from the Swedish Medical Research Council.

and constant pH were ensured by passing a stream of 99 per cent O<sub>2</sub> and 1 per cent CO<sub>2</sub> through the solution and by adding a suitable amount of NaHCO<sub>3</sub>. Normal colloid osmotic pressure was maintained by the addition of 3 per cent Dextran (GRÖNWALL & INGELMAN, 1945) to the solution. Determination of the mechanical tension has been performed with a condenser myograph according to BUCHTHAL (1942). This consists of a fixed and a movable condenser plate. The movable plate is connected with a pair of micro-tweezers which hold one end of the muscle bundle. When the muscle contracts the distance between the two condenser plates is diminished and changes in capacity are induced which are registered by means of a high frequency circuit, d. c. amplifier and electrostatic oscillograph. The changes in electrical tension due to variations in capacity are proportional to the variations in mechanical tension of the cardiac muscle.

The muscle was placed in a chamber containing 0.15 ml Ringer solution and was held by two pairs of micro-tweezers which were used as stimulating electrodes. To reduce polarisation tweezers made of silver were used. Excitability and strength-duration curves were determined with rectangular current pulses of variable duration from a stimulator of multivibrator type. Control of the electrical a. c. conductivity between the electrodes ensured that changes in excitability were not due to changes in resistance in the solution + muscle. The measuring frequency was 1,000 cycles per sec. For details we refer to a previous paper (LUNDIN 1944).

The experiments were performed at 20° C, the temperature being checked thermoelectrically.

#### Preparation of Substances.

*Adenosine triphosphate (ATP).* The ATP was prepared from rabbit muscle as the Ba salt by the method of D. M. NEEDHAM (1942), and as the neutral or acid Ba salt and the Ca salt by the method of S. E. KERR (1941). No Ba salts and only Ba-free reagents were used in the preparation of Ca ATP.

A solution of Na ATP was prepared from the Ba salt by addition of the calculated amount of sodium sulphate to the Ba salt either suspended in water or dissolved in dilute hydrochloric acid.

The Ca ATP was converted into the Na salt by the addition of the calculated amount of sodium oxalate to the solution of Ca ATP in dilute hydrochloric acid. Both the isolated Ba and Ca salts and the final Na ATP solutions were analysed for N and for total and 7' P according to the method of FISKE and SUBBAROW (1925) in the modification of SCHEEL (1936). The purity of each substance was at least 98—99 per cent.

*Creatine phosphate* was prepared by the method of ZEILE (1938) by phosphorylation of creatine with POCl<sub>3</sub>. The isolated Ca salt was first purified according to ZEILE and then converted into the Ba salt, which was recrystallised repeatedly from water-methanol according to DEUTSCH & al. (1938). Analysis: P, 8.4 %, N, 11.3 %; C<sub>4</sub>H<sub>8</sub>O<sub>5</sub>N<sub>3</sub>PBa.

$\cdot \text{H}_2\text{O}$  requires: P, 8.5 %, N, 11.5 %. A solution of sodium creatine phosphate was prepared by precipitation of Ba with the calculated amount of sodium sulphate from the solution of the Ba salt in water.

*Acetyl phosphate.* The substance was prepared according to LYNEN (1940) as the silver salt (P, 8.9 %; calc. for  $\text{C}_2\text{H}_3\text{O}_6\text{PAg}$ : P, 8.8 %). A solution of the sodium salt was prepared by addition of the calculated amount of NaCl to the suspension of the silver salt in water.

*Sodium triphosphate* ( $\text{Na}_5\text{P}_3\text{O}_{10} \cdot 5\text{H}_2\text{O}$ ). The substance used contained c. 85 per cent  $\text{Na}_5\text{P}_3\text{O}_{10} \cdot 5\text{H}_2\text{O}$  and 12 per cent sodium orthophosphate.

*Sodium pyrophosphate, sodium orthophosphate, sodium metaphosphate* and the different *barium, calcium, magnesium and strontium salts* used were of analytical purity.

All substances were applied in iso-osmotic solutions, by replacing part of the NaCl + water in the Ringer solution by an equivalent amount of staple solutions, of the substances tested adjusted to pH 7.3.

## Results.

1. *Effect of Ba.* Of different inorganic ions investigated Ba showed a quite unique effect independent of its application as chloride, nitrate or acetate.  $\text{BaCl}_2$  regularly initiates automatic activity, the threshold concentration being  $0.3 \cdot 10^{-7}$  mol/ml. The absolute amount of Ba added is 0.7  $\mu\text{g}$ . The automatic activity is preceded by definite changes in irritability, which also manifest themselves in the strength-duration curve (Fig. 1).

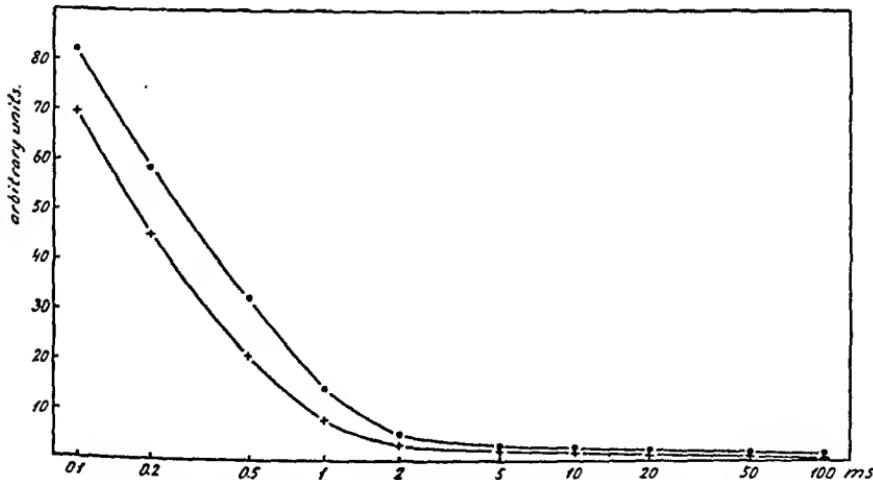


Fig. 1. Strength-duration curve of cardiac muscle preparation in Ringer solution (—●—●—) and after application of  $\text{BaCl}_2$  (—×—×—).

Ordinate: strength of stimuli in arbitrary units.  
Abscissa: time in ms.

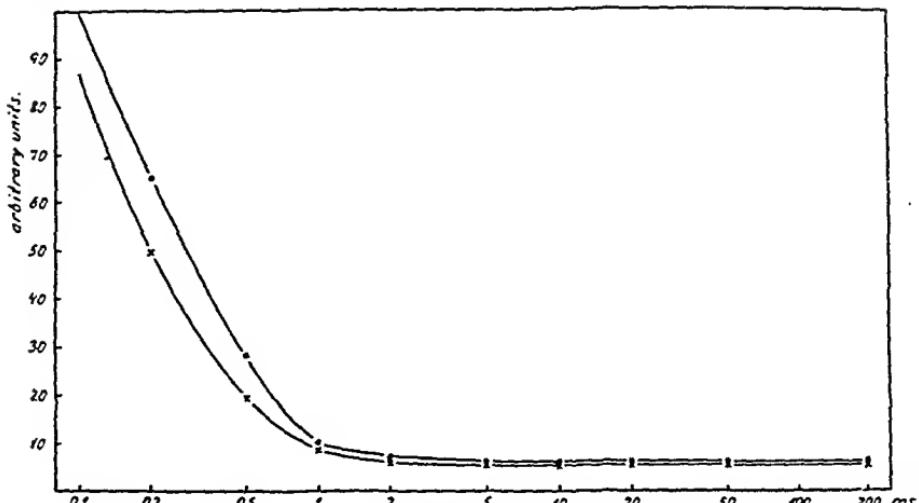


Fig. 2. Strength-duration curve of cardiac muscle in Ringer solution (—●—●—) and after application of Na triphosphate (—+—+—).

Ordinate: strength of stimuli in arbitrary units.

Abscissa: time in ms.

With a duration of the stimulus of 2 ms the threshold decreases approximately 30 per cent in the course of 2–3 minutes (Fig. 3) followed by automatic activity.

2. *Strontium* also initiates automatic activity though the threshold amounts necessary ( $5 \cdot 10^{-6}$  mol/ml), are approximately fifty times higher than with Ba. *Calcium* and *magnesium chloride* in the same concentrations have no effect on excitability, but the addition of  $\text{CaCl}_2$  ( $10^{-6}$  mol/ml) increases the strength of contraction up to 45 per cent.

3. *Adenosine triphosphate* prepared in different ways and applied as the sodium salt in concentrations of  $1.8\text{--}3.6 \cdot 10^{-6}$  mol/ml had no effect on the excitability and contractility of cardiac muscle, while highly active in experiments on skeletal muscle.

4. Application of *creatine phosphate* in concentrations of  $3.6\text{--}7.2 \cdot 10^{-6}$  mol/ml causes a decrease in the mechanical response of approximately 50 per cent, five minutes after addition of the solution. The simultaneous increase in excitability (duration of stimuli 2 ms) amounts to approximately 30 per cent.

5. *Acetyl phosphate* in concentrations of  $3.6\text{--}7.2 \cdot 10^{-6}$  mol/ml is without effect on excitability and contractility.

6. *Sodium triphosphate* and *pyrophosphate* in concentrations of  $3.6\text{--}7.2 \cdot 10^{-6}$  mol/ml cause in the course of a few minutes a considerable reduction in contraction tension, and in most cases

contractility disappears completely. This effect is reversible and after washing out with Ringer solution for 1-2 minutes, the original strength of contraction is quickly restored. Excitability after triphosphate for a duration of the stimuli of 2 ms increases 25-70 per cent depending on the concentration applied. The increase is most pronounced with short stimuli as seen from the strength-duration curve in Fig. 2.

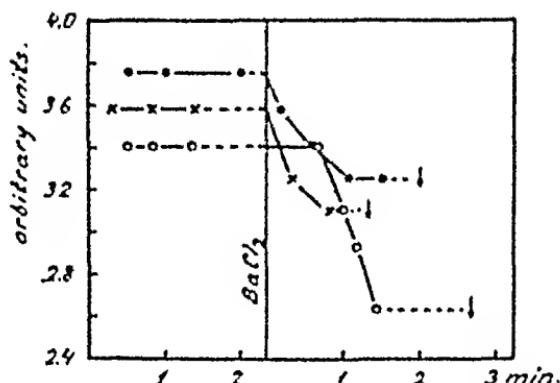


Fig. 3. Excitability of cardiac muscle preparation after m/10,000 (—●—●—), m/20,000 (—+—+—) and m/25,000 (—○—○—) BaCl<sub>2</sub>. The arrow denotes start of automatic activity.

Duration of stimuli 2 ms.

Ordinate: strength of stimuli in arbitrary units.

Abscissa: time in minutes.

7. Sodium metaphosphate in concentrations of  $3.6-7.2 \cdot 10^{-6}$  mol/ml also causes a strong decrease in the strength of contraction, while orthophosphate affects mechanical tension only slightly. Excitability for stimuli of 2 ms duration is improved after pyrophosphate and metaphosphate and is more pronounced with still shorter stimuli of 0.2 ms duration, when even orthophosphate improves excitability.

### Discussion.

Although the stimulating effect of Ba on smooth and striated muscle is well established, observations with regard to its action on cardiac muscle are rather contradictory. It has often been compared with the action of digitalis as many authors describe its effect as a decrease in frequency and an increase in mechanical contraction tension. There is unanimity with regard to the effect of large amounts of Ba, evoking a systolic contracture. Small doses are stated to increase frequency (SALANT & KLEITMAN,

1922) or to have the reverse effect (LIOTTA, 1924, TOCCO-TOCCO, 1924). It has been used therapeutically in cases of auriculo-ventricular dissociation (COHN and LEVINE, 1925), where it apparently has a regulating effect.

KISCH (1927) has investigated the action of the alkaline earth cations on the initiation of impulses in the frog's heart and found Ba more active than the other alkaline earths in facilitating the physiological stimuli. Our investigations establish the specific action of the barium ion on cardiac muscle, releasing an automatic activity of several minutes duration, when applied in minute amounts.

Fig. 4. Antagonistic effect of barium to acetylcholine on the Straub heart preparation. Arrow 1 denotes the addition of acetylcholine 1 : 10<sup>7</sup>, then washing out with Ringer solution and at arrow 2 addition of BaCl<sub>2</sub> (10<sup>-7</sup> mol/ml) and arrow 3 renewed addition of acetylcholine 1 : 10<sup>7</sup>.

(ABDON, 1942) also can be produced by small amounts of BaCl<sub>2</sub>. We find in the Straub heart preparation that BaCl<sub>2</sub> in a concentration of 10<sup>-7</sup> mol/ml counteracts the effect of acetylcholine on the frog's ventricle (Fig. 4).

### Summary.

1. Barium salts in minute amounts initiate automatic activity in the cardiac muscle, the threshold concentration being  $0.3 \cdot 10^{-7}$  mol/ml. The automatic activity is preceded by a definite improvement in excitability. This effect is specific to Ba; Ca and Mg are inactive and for Sr, the threshold concentration is approximately fifty times higher than for Ba.
2. Adenosine triphosphate has no effect on cardiac muscle in concentrations of  $1.8 - 3.6 \cdot 10^{-6}$  mol/ml.
3. Creatine phosphate in concentrations of  $3.6 - 7.2 \cdot 10^{-6}$  mol/ml causes a decrease in the mechanical response of c. 50 per cent.

4. Inorganic triphosphate, pyrophosphate and metaphosphate in concentrations of  $3.6-7.2 \cdot 10^{-6}$  mol/ml cause a considerable reversible fall in contraction tension of cardiac muscle. Orthophosphate produces a slight decrease in the strength of contraction.

5. Barium chloride in a concentration of  $10^{-7}$  mol/ml counteracts the effect of acetylcholine on the frogs ventricle in the Straub heart preparation.

The authors wish to express their gratitude to Mr. F. BUCHTHAL, M. D., for valuable advice.

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## The Principle of Evacuation of the Stomach in Infants and Prematures.

A non-roentgenologic study.

By

STEPHAN VENDEL.

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For several decades the motor function of the stomach has been the object of comprehensive investigations (cfr. CATEL 1936), but despite the minute observations of many experienced investigators the separate results will be seen to differ so much from each other that it is still possible only to speak about the emptying of the stomach in the most general terms. The time of evacuation varies very considerably in different individuals, and even in the same individual on repeated examination. A fairly great variance is found between different groups, *e. g.* when comparing a number of works of the medical literature, as did BOUSLOUG (1935).

Therefore, SMITH (1945), is fully justified when he writes as follows: "No uniformity is to be expected. The stomach empties with unpredictable variability", but it is difficult to imagine that this should really apply to infants who in most of their other vital functions display so marked a regularity. It has been tried to explain the variations found from the affectibility of the stomach by its reflex and hormonal regulation. On the other hand, in CATEL's statement (vol. I, page 121) the following description of the gastric peristalsis is found: . . . "eine Präzision der rytmischen Tätigkeit, die überrascht . . . kaum um Bruchteile differierend"!

Nearly all investigations are roentgenologic. In most cases a contrast medium was employed, but a few investigators have wanted to safeguard against possible methodic errors caused by this examination and made the transillumination without using barytes, as did BEHRENDT (1923) and BESSAU, ROSENBAUM and LEICHTENTRITT (1921). The predominant use of the roentgenologic method must be attributed to the fact that it has been believed that all factors changing the natural course of the evacuation could be avoided: The process can be followed without interference and the peristalsis observed, and it can be seen directly when the last residue leaves the stomach; and, if desired, these impressions can be fixed on a roentgen film. With some practice a certain perception of the course is obtained from the extent and density of the roentgen shadow: In some cases it will be seen that to begin with the stomach empties more rapidly, later on far more slowly while, in other cases, the reverse seems to be the case. The flaw of the roentgenologic method is that, besides making the peristalsis visible (and showing, when the first portion of the meal enters the duodenum, if a barium meal has been given) it gives only one exact item of information: The time when the stomach gets empty — and that with so great variations that, in spite of everything, the method must be suspected of being encumbered with great sources of error. It gives no useful information about the entire intervening part of the evacuation curve, and cannot do so either until methods have been prepared like that used in determination of the volume of the heart *in vivo*.

Both evacuation curves have their adherents among physiologists. The divergence of opinions is perhaps best illustrated by the following quotations: STARLING: "As emptying proceeds, the rate of evacuation is readily slowed down by influences, probably reflex, but also in part hormonal . . .", and by WIGGER (1934): "The emptying rate of the stomach increases progressively from the onset to the completion of digestion."

Only through quantitative determinations in the course of the evacuation will it be possible to elucidate which type of emptying is the right one. Such determinations are found only exceptionally in the literature, but WILSON's statements afford an example. Graphic representation of his table values conveys the impression that the nature of the food itself determines the type of evacuation: Raw egg-white represents the former — the

"concave" curve; whereas egg-yolk with bacon results in the "convex" shape. By fluoroscopy an estimate was made of the percentage of the barium remaining in the stomach after  $1\frac{1}{2}$ , 3 and  $4\frac{1}{2}$  hours.

As the times of evacuation in infants displayed so wide limits of variation, even a rough method with comparatively great experimental errors will be justified if only it elucidates one new feature of the evacuation of the stomach, be it either so that the variations have been due to the natural conditions or have simply reflected hitherto unnoticed methodic errors in the roentgen examination.

In the present investigation our aim has been to follow the entire course of evacuation, and not to be content with determining the initial and terminal points of the curve. The method we chose was simple use of an ordinary stomach tube (size 12 to 14) and a graduated Record syringe of 50 or 100 cc. The rate of evacuation is followed by emptying the stomach at regular intervals (by means of suction with the syringe), measuring the quantity removed and then injecting it again into the stomach until it is time again to remove it through the stomach tube. Unfortunately a permanent tube cannot be used in infants, the tube has to be introduced again for each determination. It would moreover be desirable to be able to empty the stomach completely and refill it without changing its volume in the course of the procedure, to avoid stimulation of the peristalsis. This is possible only when using a Miller-Abbott's double channel tube, which is of too coarse a caliber for this examination in small infants. Besides it proved possible to arrive at satisfactory results without these precautions and then to reduce the number of necessary introductions of the tube, so that disturbances originating from the use of the stomach tube were completely eliminated from the result of the experiment.

The basis of this communication is a series of 110 experiments, carried out on 27 children, aged 1 week—11 months. Of these 3 were examined shortly after gastrointestinal disturbances, and in 5 cases pharyngitis was present. In the rest of the children only disease of minor importance to gastric function, or no disease at all was present. A number of formulae were given: mother's milk, acidified milk, etc., to exemplify any commonly used formula, but the material hitherto collected being by this reason too small and too heterogeneous to give exact informations of the various

emptying times, yet will allow certain preliminary conclusions about the process of evacuation:

1) In most infants the stomach empties completely regularly, despite the disturbing influence of the stomach tube; the evacuation proceeds with the precision of clockwork till at least  $\frac{2}{3}$ , or sometimes  $\frac{9}{10}$  (or the whole) of the meal, has left the

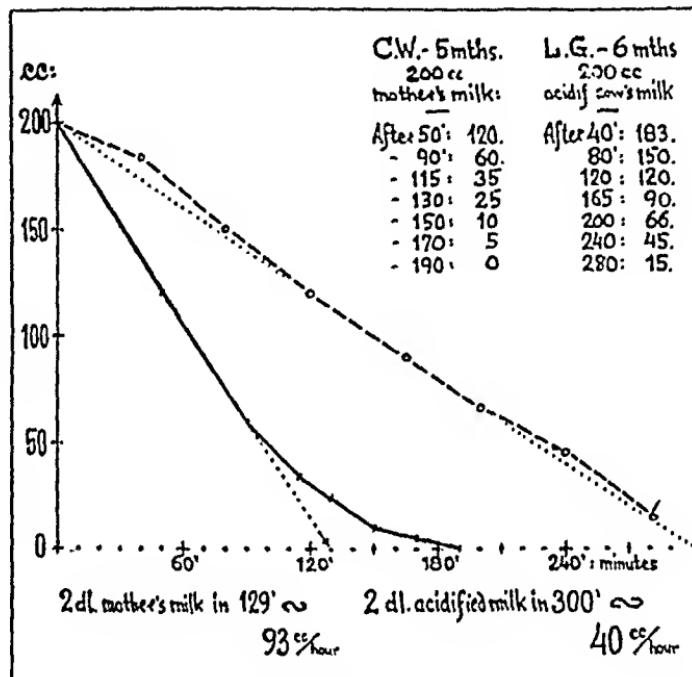


Fig. 1.

stomach, then its emptying rate decreases somewhat. With this qualification it can be said that the stomach empties a constant amount per time unit almost during the whole of the meal. In graphic representation it means a rectilinear curve. If this line is elongated (extrapolated) till it intersects the zero line, the result will be a time which might be termed the ideal emptying time for the meal in question and which it would be possible to express in minutes. The number of minutes required by the stomach to empty 100 cc is an exponent of the emptying rate, but in comparisons between the emptying times of children at different ages the inverse proportion, *i. e.* the amount emptied per hour, is advantageously used as emptying coefficient ( $= e_t$ ). The quantity  $e_t$  varies inconsiderably as long as it is not determined for very

large or very small meals: the linear curve applies within wide "normal limits". This conception of the emptying process is seen to be in full harmony with the results of CRIDER and THOMAS' investigations: "After larger meals the emptying time is greater in most subjects than after small meals; *e. g.* doubling a small test meal may increase the time by 17 per cent, and trebling it

may increase it by 38 percent." An approximate prolongation of 100 and 200 per cent should have been less confusing than these 17 and 38 per cent, but we have to reckon in most cases with a course of emptying, different from the "ideal". The reasoning is elucidated best by the curve (fig. 2). In this connection also BOUSLOUGS statement —

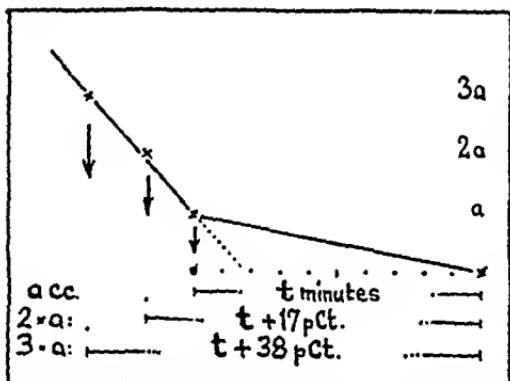
Fig. 2. Result of CRIDER and THOMAS' investigation graphically reproduced: a curve resembling the last part of our curves.

"it would appear that feeding before the stomach was empty did tend to lengthen the emptying time" finds its natural explanation. The same holds good of the observation that debile infants would empty their stomachs more rapidly than normal infants — they get smaller meals!

2)  $c_t$  varies fairly moderately in the individual child from time to time, there is a somewhat greater variation between individuals within the same age group. Our material does not yet allow the exact determination of the variance ( $\sigma$ ).

3) On comparison between the mean figure for  $c_t$  of the different age groups it is seen that this constant increases regularly month after month for each kind of food in proportion to the growth of the child. If  $c_t$  is, therefore, divided by the normal weight for the age in question, a quotient results which is nearly constant all through infancy for a definite sort of food or milk mixture, whereas in prematures it is found to be decreased in proportion to the debility of the child, expressing its functional insufficiency.

4) This reduced emptying coefficient is of typical magnitude for each kind of food. The ratio between these coefficients is found to be fairly unaltered on determination of the  $c_t$  of the



single milk mixture in the individual child. Bearing in mind the experience of previous researchers about emptying times, it is astonishing to see how moderate the deviations in this ratio are as compared with the mean figure.

Exact, statistically tenable determinations of these figures are being prepared and will be published in a subsequent paper, in which reasons of individual variations will also be discussed; the paper will also comprise investigations into the conditions which, in our opinion, determine the different emptying coefficients for the various kinds of food in infants.

### Summary.

By roentgen only the initial and final points of the evacuation curve of the stomach can be determined exactly enough. To follow the entire course of evacuation another technic was applied: the volume of gastric contents being measured at regular intervals by aspiration through a stomach tube. It was demonstrated that the stomach in infants empties with extreme regularity, following mostly a rectilinear curve, the emptying rate depending upon age and the constituents of the meal, and the variations tending to be much more confined than previously accepted.

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ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 11. SUPPLEMENTUM XXXIII.

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CONTRIBUTIONS TO  
THE KNOWLEDGE OF THE EFFECT  
OF EXOGENOUS INSULIN ON  
THE GLYCOGEN STORAGE  
OF NORMAL ANIMALS  
AND A SURVEY OF FACTORS NORMALLY  
INFLUENCING THAT STORAGE

By

ÅKE SWENSSON

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Stockholm 1945



*Till mina föräldrar*



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## PREFACE

The present investigation was carried out at the Histological Department of Karolinska Institutet at Stockholm during the years 1942—1945.

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The technical work in this investigation has been carried out with great thoroughness and untiring zeal by Miss ANN-MARIE BÅNG and by my wife ANNALISA SWENSSON, to whom I wish to express my gratitude, as also to Miss MAJ BERGHMAN, who prepared the diagrams.

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Stockholm, October 1945.

ÅKE SWENSSON

# INTRODUCTION

Even a superficial study of the reports in the literature regarding the effect of insulin on the glycogen content of the liver and muscles shows that there is much confusion on this subject. Ever since CLAUDE BERNARD in 1855 detected glycogen in the liver, this substance has been subjected to much research and, as soon as serviceable insulin preparations were obtained, a number of investigators began to study their effect on the liver and muscle glycogen. Since the beginning of the twentieth century glycogen metabolism had in fact assumed a central place in metabolism research. As investigators at first did not realize the amount of the biological variation, they contented themselves with very few experiments, which is one of the reasons why the literature on the subject is full of contradictory statements. As the importance of even minute variations in the experimental conditions was not realized, incommensurable experiments were compared, and the discussion was often based on comparisons with the results of other investigators, even under conditions where they were not applicable. Other publications on the subject are marred by fundamental errors in the discussion and appraisement of the results.

Despite the immense amount of labour bestowed on the study of this question, however, it has still been found impossible to arrive at any consensus of opinion.

The views expressed in the literature in regard to the effect of insulin on the glycogen depots of the body in fact show considerable discrepancy. Thus, v. MEYENBURG (1924) states: »Zusammenfassend möchte ich also sagen: Es scheint sicher gestellt dass

unter der Insulinwirkung eine Glykogenstapelung in der Leber stattfindet, wenigstens bei einigen Tierarten. Ob alle bei der Insulinhypoglykämie aus dem Blut verschwindende Zucker in der Leber konzentriert wird, bleibt dagegen unsicher.»

MACLEOD (1924) states: »There can be no doubt that insulin causes a rapid reduction in the amount of glycogen in the liver and may cause it almost, if not entirely, to disappear from the muscles.»

GREVENSTUK and LAQUEUR (1925 b), after a thorough survey of the literature of that time, consider it proved that insulin induces »bei normalen, gut genährten, wie hungernden Tieren Abnahme des Glykogens, Zunahme des Glykogens bei pankreasdiabetischen Tieren, aber wahrscheinlich auch bei normalen, wenn gleichzeitig mit dem Insulin Zucker gegeben wird». They add that they cannot state for certain whether insulin has any effect on the glycogen content of the muscles.

ISAAC and SIEGEL (1928) sum up their views as follows: »Unter Einwirkung zugeführten Insulins wird Zuckerverbrennung und Glykogensynthese in der Muskulatur beschleunigt». As regards the liver, they state: »Das Wesen der Insulinwirkung, soweit dieselbe heute erklärbar ist, besteht in einer Beschleunigung des gekoppelten Prozesses von Zuckerverbrennung und Glykogensynthese».

STAUB (1930), summing up, states: »Der normalphysiologische Insulineffekt am gesunden, glykogenarmen Organismus besteht demnach in Beschleunigung der Glykogenbildung im ganzen Organismus und wahrscheinlich auch in der Leber. Mittlere toxische Dosen vermindern nur den Glykogengehalt der Leber und reichern denjenigen des übrigen Organismus etwas an; es kommt aber im ganzen zu einem Glykogendefizit. Hochtoxische Insulingaben verringern sowohl Leber- wie Muskelglykogengehalt erheblich».

C. F. CORI (1928) considers that a marked increase of oxidation and storage of sugar is found in the peripheral tissues after the

injection of insulin, but that the liver glycogen content is greatly reduced. And this occurs even if the animal has carbohydrates available in its intestine in the meantime. He comes to the following conclusion: »This discussion shows that our information concerning the influence of insulin on the carbohydrate metabolism of the liver is still very scanty. There are no convincing experiments on record which would show a direct influence of this hormone on either glycogen synthesis or glycogenolysis. The experiments on the perfused liver of mammals and of cold-blooded animals did not yield results that permit of a definite interpretation. In normal fasting animals the majority of the investigators observed a decrease in liver glycogen after the insulin injection . . .»

It appears from the foregoing discussion that further work is needed to elucidate the relation between insulin and the carbohydrate metabolism of the liver.

GEILING, JENSEN and FARRAS (1937), summing up, state: »In contrast to its action upon the diabetic, insulin usually decreases the glycogen stores of the normal fed and starved animal. Its effect upon the liver and other glycogen depots is, however, a complex matter, difficult to interpret in the light of our present imperfect knowledge concerning the mechanism of insulin action.»

LUNDSGAARD (1939) writes: »Today, just as was the case immediately after the discovery of insulin, the question of how insulin works can be answered only by reference to the directly observable effect of insulin in the animal organism: a reduction of the blood sugar concentration. If one is asked as to what causes this reduction of the blood sugar concentration, the only positive answer is that the effect, partly at any rate, is the result of an acceleration of the rate of migration of glucose into the striated muscle fibres from the blood and an interconnected acceleration of the glycogen deposition in the muscles. It is doubtful if insulin has a corresponding effect in the liver.»

GERRITZEN (1942) states it to be the general view in modern times that »insulin promotes the fixation of glycogen in the liver, or, perhaps better expressed: checks the flow of glucose into the blood, thus giving rise to a larger content of glycogen in the liver. Insulin promotes the deposition of glycogen in the muscle.»

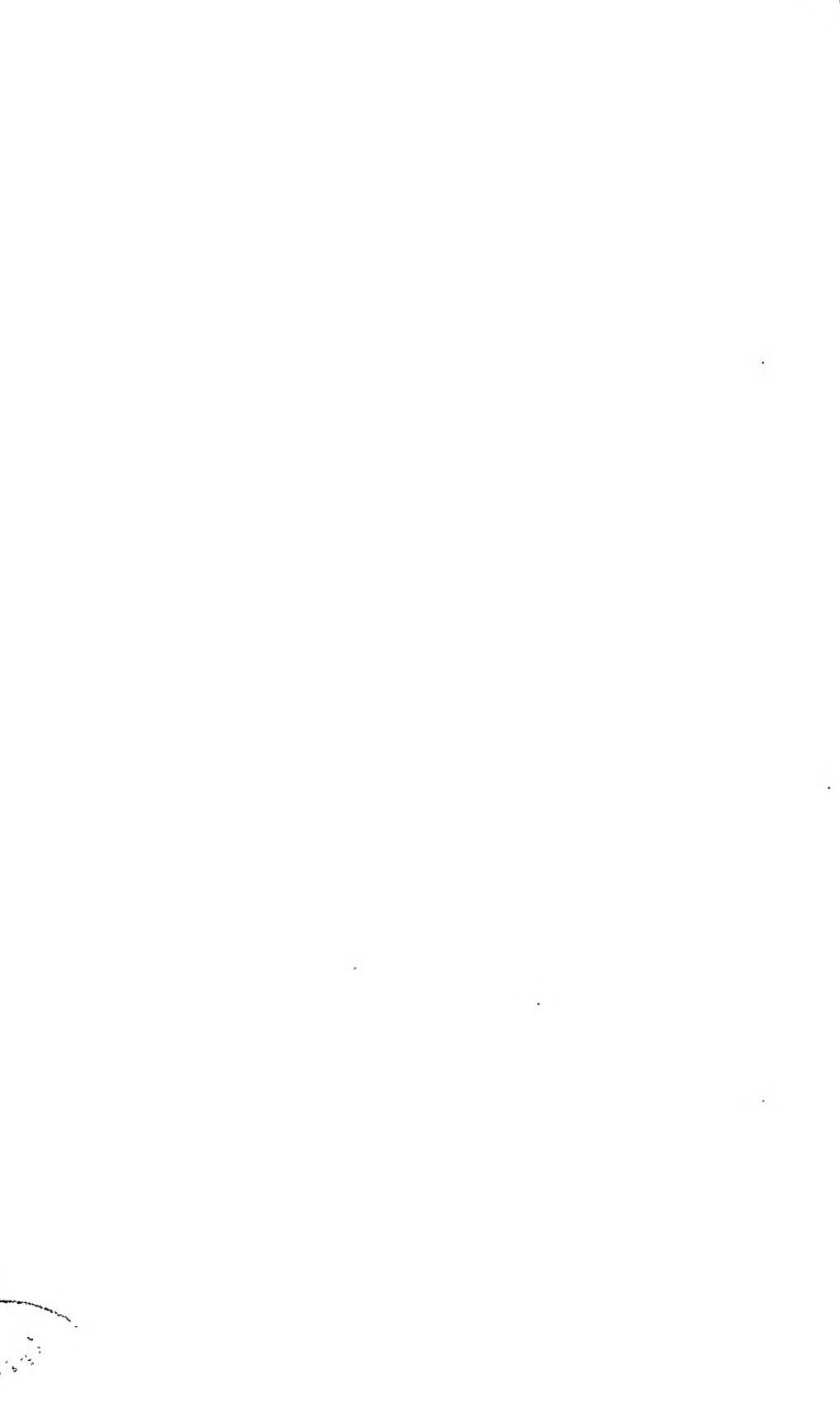
BEST and TAYLOR (1943) state: »In the normal animal one of the most clear-cut effects of insulin is the increase in the rate of glycogen deposition in the muscle which it produces, but it has not been demonstrated in any normal adult animal that insulin increases the level of liver glycogen. The increase in the livers of young rats is due to a secondary liberation of adrenaline. In the normal adult animal there may be an actual loss of glycogen from the liver when insulin is administered. This is due to the accelerated glycogen deposition in muscle and the increased rate of oxidation of sugar.»

A systematic review of the immense number of investigations published on this question falls beyond the scope of this work. Here it must suffice to mention some of the works most cited in the literature as well as those which have an important bearing on the discussion.

The present investigation is intended to obtain reliable data regarding the effect of insulin on the glycogen stores of the body, when it is supplied in excess to normal animals. This would provide a firm basis for continued studies on the effect of insulin.

The investigation falls into two parts: 1) Study of the conditions for comparative glycogen investigations, *i. e.* a study of the factors affecting the glycogen storage in normal animals and an inquiry as to how the least possible variation in these values can be attained. 2) Study on the effect of insulin on the glycogen reserves of the body in normal animals.

PART I  
METHODS



## CHAPTER I.

### The glycogen analysis method

#### A. Description of the method.

The glycogen determinations in the present investigation were made in accordance with the modification of PFLÜGER's method indicated by SJÖGREN, NORDENSKJÖLD, HOLMGREN and MÖLLERSTRÖM (1938).

The analysis is made in the following manner:

I. *Liberation of the glycogen*: Centrifugal tubes are charged with 2—3 cc 30 % KOH and are heated in a boiling water bath. Suitable pieces of the organs are weighed rapidly on a torsion balance with a sensitivity of 10 mg and are plunged into the hot potash lye. If large pieces of tissue, over 1 gram, are taken, the amount of potash lye must be increased in proportion. Boiling for 30 minutes, with frequent shaking.

II. *Precipitation and washing of the glycogen*: After cooling of the tube, 0.5 cc of a saturated sodium sulphate solution is added as well as *spiritus concentratus* in sufficient amount to bring the grade of alcohol up to 75—80 %. Afterwards heating in a water bath to boiling point, cooling in running water and centrifuging. The clear mother-lye is cautiously poured away. The precipitate is dissolved in a small amount of distilled water (2—3 cc) and the precipitation with sodium sulphate, alcohol and boiling is repeated. Cooling, centrifuging and decanting. The precipitate is again dissolved in water and precipitation is once more made, now without the addition of sodium sulphate, with alcohol solely.

III. *Hydrolysis*. The precipitate is dissolved in 3 cc of water and exactly 3 cc of 10 N sulphuric acid is added. Hydrolysis is effected by boiling in a water bath with an air-cooler for 30 minutes.

**IV. Neutralization and dilution:** After hydrolysis the solution is neutralized with 3 N NaOH, with phenolphthalein as an indicator. The sample is diluted in a volumetric flask precisely to the required volume.

**V. Determination of the amount of glucose in the diluted sample:** Exactly 5 cc of the solution is mixed in a pyrex tube with exactly 5 cc of SCHAFFER-SOMOGYI's reagent. The tube is plunged into a boiling water bath; is allowed to boil for precisely 15 minutes, and is then quickly cooled in cold running water. Thereupon 2 cc of a solution containing 2.5 % of potassium iodide and 2.5 % of sodium oxalate is added, and then 5 cc N H<sub>2</sub>SO<sub>4</sub>. The sample is shaken now and then, and after 10 minutes is ready for titration with N/200 sodium thiosulphate, with starch as an indicator.

The analysis sample, after titration, should contain 0.5—2.0 mg glucose. If it contains more, the method will give too low values, and the analysis will have to be repeated with a smaller amount of test-solution (4—3—2—1 cc) and, instead, addition of 1—2—3—4 cc distilled water. Should too high glucose values be obtained even with 1 cc test-solution, a new dilution must be made.

The amount of glucose is found by readings on a comparison curve, which is obtained by measuring in four tubes 2, 3, 4 and 5 ml of a freshly prepared solution of exactly 50 mg pure glucose per 100 cc, adding 3, 2, 1 and 0 cc of water, so that all the tubes contain 5 ml, and then 5 cc SCHAFFER-SOMOGYI's reagent to each tube; analysis as above. The amounts of thiosulphate obtained are marked on a curve with the amount of glucose in the test-solution along the x-axis and the amount of thiosulphate along the y-axis. At least three of the values obtained should lie along a straight line.

The value read off on the curve indicates the amount of glucose in the analysis test. From it the amount of glucose in the organ sample is computed. The glycogen value is obtained by multiplication with the factor 0.927 (PFLÜGER).

According to SJÖGREN, NORDENSKJÖLD, HOLMGREN and MÖLLERSTRÖM, this method gives results with an accuracy of  $\pm 5\%$ , »... welche als die zur Zeit optimale anzusehen ist». Some earlier authors have spoken of analyses with a precision of  $\pm 2\%$ .

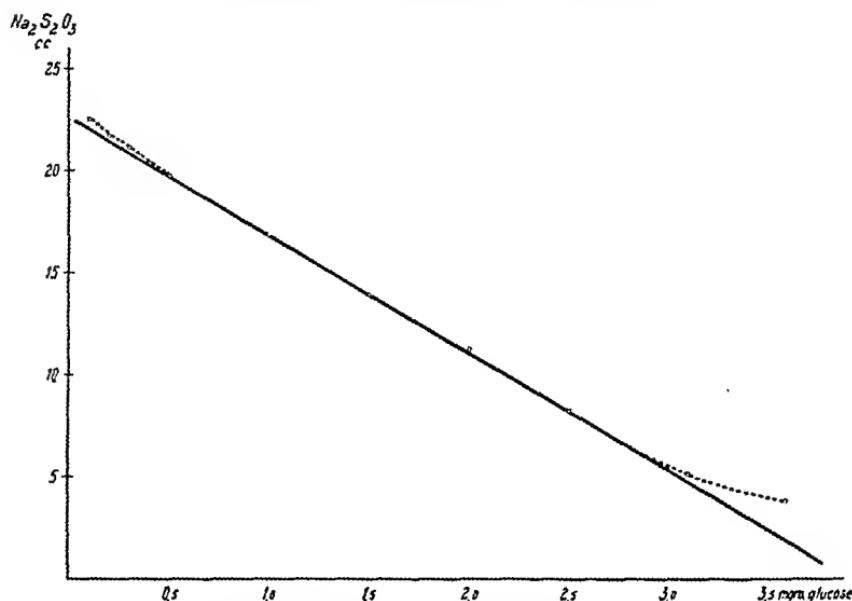
This, however, is considered by SJÖGREN and his co-workers to be a considerable overestimate of the exactitude of the method. As for the yield, it is not mentioned at all by Sjögren *et alios*. For a similar method, SAHYUN (1931) reports a yield of about 100 % on analysis of glycogen amounts ranging between 5.0 and 160 mg. His series, however, are very small, and we cannot gather from his published report whether the figures are based on the results of individual analyses, or whether they are averages for serial analyses. He gives no figures for the deviation. — Similar figures are reported by other authors.

It seems *a priori* improbable that a chemical method of this nature should give a yield of 100 per cent. Evidently, none of the various processes involved in the method could be carried on entirely without loss, though the loss may be very small. In the present investigation therefore the accuracy of the method has been tested with a view to ascertaining (1) the percentage of the yield, (2) where in the course of the analysis a possible loss occurs and (3) the constancy of the results, which, of course, is a matter of special importance in practice.

## B. The testing of the method was made in the following manner.

1. *Testing of the glucose analysis method.* As the glucose determination is based on the reading of a comparison curve, and as that curve is traced from four points by analysis of four samples containing known amounts of glycogen, it is essential to ascertain whether the straight line thus obtained can actually be applied to higher and lower glucose values than those used in the construction of the curve. Another important matter is the determination of the accuracy of the individual analysis. These two factors were determined by serial analyses of pure glucose. Different amounts of glucose solutions with a known concentration were analyzed. A curve was drawn on the basis of the average consumption of thiosulphate for the amounts of glucose ordinarily used in the construction of such curves. This curve was extended in both directions. The values for the consumption of thiosulphate with the

*Fig. 1. Diagram showing required volume of  $\text{Na}_2\text{S}_2\text{O}_3$  when known quantities of pure glucose are determined.*



— curve drawn as described in the text.

- - - - - curve based on estimations including excessively small or large quantities of glucose.

other analyzed amounts of glucose were then inserted. See the diagram Fig. 1 and the table Fig. 2.

Figs. 1 and 2 show that the curve for the consumption of thiosulphate is linear as regards glucose amounts ranging between 0.5 and 2.5 mg. Whether the amounts of glucose in the samples analyzed are larger or smaller than these limit values, relatively larger quantities of thiosulphate will be consumed. Thus, if a comparison curve is traced in the usual way, the glucose readings will be too low. As will be seen from the table in Fig. 2, the standard deviation of the consumption of thiosulphate in fact varies in respect of different amounts of glucose. It shows a marked increase when the amount of glucose reaches 2.5 mg or more. However, if we glance at the column showing the deviation expressed in glucose, it will be seen that the variation in the result of the analysis keeps fairly constant, though it rises somewhat for the larger amounts of glucose. If, on the other hand, this deviation is placed in relation to the amount of glucose in the sample, *i. e.* if we look

Fig. 2. Table. Determinations of known quantities of pure glucose. Control of the variability of the method of glucose estimation.

Quantity of glucose added. Mgm.	Number of determinations	Required vol. of $\text{Na}_2\text{S}_2\text{O}_3$		Standard deviation of required vol. of $\text{Na}_2\text{S}_2\text{O}_3$ given as corresponding quantity of glucose according to			
				the correct curve. Fig. 1	the constructed curve. Fig. 1	Glucose mgm	Per cent of glucose added
		cc	$\sigma_x$			Glucose mgm	Per cent of glucose added
0.1	14	22.5	0.5	0.07	70	0.08	80
0.2	14	21.7	0.6	0.09	45	0.10	50
0.3	14	21.1	0.6	0.08	27	0.10	33
0.4	14	20.3	0.5	0.08	20	0.08	20
0.5	14	19.7	0.6	—	—	0.10	20
1.0	14	16.8	0.5	—	—	0.08	8
1.5	14	13.9	0.6	—	—	0.10	7
2.0	14	11.2	0.6	—	—	0.10	5
2.5	14	8.2	0.8	—	—	0.18	5
3.1	14	5.1	1.2	—	—	0.21	7
3.6	14	3.8	0.8	—	—	0.13	4

at the standard deviation in per cent of the mean, it will be found that it is much larger for small amounts of glucose, 0.5—0.4 mg and under. As regards other analyzed amounts of glucose, it is fairly constant.

As is clearly indicated by these results, the analyses should be so adjusted that the amounts of glucose in the samples analyzed range between 0.5 and 2.5 mg. This in fact was the rule adopted throughout the present investigation, with the exception of a few series where, owing to the low glycogen content in the organs, it was not found possible to reach such high values even when the whole organ was analyzed. However, a glycogen value below 0.4 mg per analyzed sample hardly ever occurred. These values too were read on the ordinary comparison curve. In fasting mice, which have a very low glycogen content, this was partly compensated by the analysis of the livers in couples.

*Fig. 3. Table showing the result of repeated glucose determinations of one and the same dilution of a given liver glycogen sample. The sample was always diluted to such a degree that the required volume  $\text{Na}_2\text{S}_2\text{O}_3$  should fall within the optimal part of the test curve.*

Number of determinations	Liver glycogen content per cent $\bar{x}$	Range	Standard deviation $\sigma_x$	Standard deviation in per cent of mean $\sigma_{\%}$
19	3.18	3.48 — 2.97	0.16	5.0
15	8.74	9.27 — 8.14	0.39	4.5
11	10.96	12.00 — 9.93	0.77	7.0
14	11.74	12.45 — 10.82	0.55	4.7

The exactitude of the sugar analysis method was also determined by analyses of liver samples. When the sample had passed through the whole analysis procedure including the dilution, a series of sugar determinations was made. The results are tabulated in Fig. 3, which gives a good idea of the accuracy of the method in practical work with different amounts of glucose. The standard deviation in per cent of the mean for all the values is fairly constant.

The sugar analysis method was controlled in yet another way, namely by double analyses of liver and muscle glycogen samples from mice. As will be seen from the table in Fig. 4, the values show throughout a very good correspondence. Nowhere is the mean difference statistically significant, that is to say, there is no

*Fig. 4. Table. Double glucose determinations of liver and body glycogen of mice.*

Organ	No of determinations	Sample I Glycogen content per cent $\bar{x}$	Sample II Glycogen content per cent $\bar{x}$	Mean difference and its standard error $\bar{d} \pm \varepsilon_{\bar{d}}$
Liver I . . . . .	23	1.08	1.05	-0.03 ± 0.08
Liver II . . . . .	24	3.33	3.33	0 ± 0.01
Muscle I . . . . .	32	0.046	0.044	-0.002 ± 0.001
Muscle II . . . . .	19	0.140	0.144	0.004 ± 0.005

Fig. 5. Table. Control of method. Determinations of known quantities of pure glycogen. Direct hydrolysis of glycogen.

Glycogen added mgm	No. of samples	Recovered glycogen mgm $\bar{x}$	Standard deviation $\sigma_x$	Standard deviation in per cent of mean $\sigma_{\%}$	Yield per cent
1.86	13	1.25	0.18	10.4	67
3.72	13	3.12	0.14	4.5	84
5.58	14	5.18	0.81	6.0	92
16.74	14	15.25	0.82	5.4	91
33.48	14	30.07	1.48	4.9	90
34.88	14	31.00	2.28	7.2	89

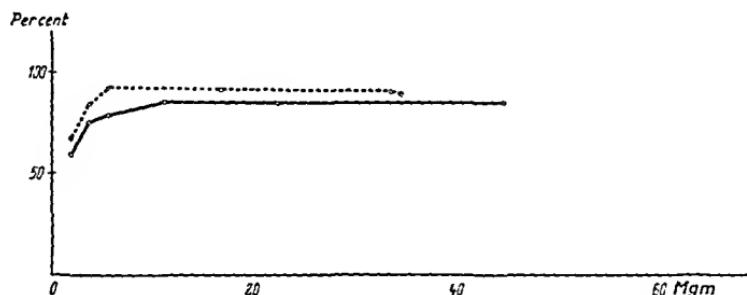
systematic difference between the values obtained in the two analyses.

These three control tests thus show that the glucose analysis method gives very reliable readings if the samples analyzed are adjusted so as to contain 0.5—2.0 mg glucose. Should the amounts of glucose be larger or smaller, too low glucose values will be obtained if a comparison curve is traced in the above-stated manner.

Besides the sugar analysis, there are two other factors in the glycogen determination that may conceivably entail losses: hydrolysis and the washing process.

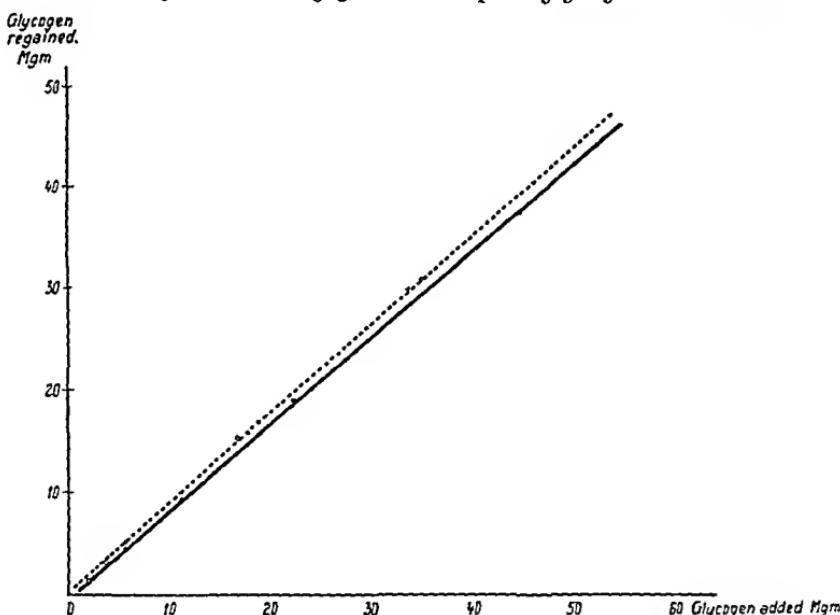
2. *The effect of hydrolysis on the yield.* In these tests, known amounts of pure glycogen per analysis were hydrolyzed and determined. All the sugar analyses were made in such a way that the consumption of thiosulphate fell, so far as possible, within the optimal part of the comparison curve. The glycogen used had a water content of 7.01 % and an ash content of 0.0029 %. In the table Fig. 5 and in the diagrams Figs. 6 and 7, the amounts of supplied glycogen refer to the »solids». As shown both by the table and by the diagrams, the results of the analysis are very uniform. Apart from the lowest amounts of glycogen, the standard deviation in per cent of the mean is constant. The losses are throughout quite small; they will, of course be relatively larger

*Fig. 6. Diagram showing yield when known quantities of pure glycogen are determined.*



----- the sample was immediately hydrolyzed.  
— the sample passed through all the procedures of the method,  
including precipitation and washing.

*Fig. 7. Diagram showing yield when pure glycogen is determined.*



if very small amounts of glycogen are analyzed, in which case the percentage yield will be poorer.

3. *The effect of the washing and precipitation processes on the yield.* The results of the analyses of pure glycogen which has passed through the whole course of analysis are tabulated in Fig. 8 and are graphically shown in the diagrams Figs. 6 and 7. The yield will be somewhat poorer than in the direct hydrolysis of

*Fig. 8. Table. Control of method. Determination of pure glycogen. The samples passes through all the procedures of the method, including usual washing and precipitation before hydrolysis.*

Glycogen added mgm	No. of determinations	Glycogen recovered mgm $\bar{X}$	Standard deviation $\sigma_x$	Standard deviation in per cent of the mean $\sigma_{\%}$	Yield per cent
1.86	14	1.09	0.21	19.3	59
3.72	14	2.81	0.50	17.8	75
5.58	14	4.39	0.83	7.6	79
11.16	14	9.26	0.65	7.0	83
22.82	12	18.97	1.28	6.7	85
44.64	12	37.73	2.10	5.6	85

the glycogen, which indicates a small loss in the precipitation and washing procedure. This agrees well with the investigations of KERLY (1930), who states that 1—2 mg of glycogen is dissolved in 100 cc 80 % alcohol. The standard deviation in per cent of the mean is also somewhat higher than in direct hydrolysis: but, when the amount of glycogen is not less than 5 mg, it remains constant, as also the percentage yield. The latter is, of course, poorer for low amounts of glycogen.

The serviceableness of the method is also indicated by the double determinations of the glycogen content in liver and muscles, which are tabulated in Figs. 20 and 21 and are discussed on p. 53.

All these control tests show that the method, if we do not work with too small amounts of glycogen, and if the analysis is adjusted so that the amount of thiosulphate consumed falls within the optimal part of the comparison curve, operates with satisfactory exactitude and gives an acceptable yield. A minor loss ensues at each step in the analysis, but nowhere any considerable loss. There is thus no particularly weak spot in the course of the analysis.

It should be noted that all the glycogen values are figures directly obtained in the analysis and have not been corrected for losses during the course of it.

### C. Other reducing substances than glucose possibly found in the hydrolysate.

In the analysis method it is, of course, intended that all other substances than glycogen should be broken down and washed away, so that after the washing only glycogen is left in the sample analyzed. Consequently, glucose should be the only reducing substance in the sample after hydrolysis.

CORI (1928) states: »The non-sugar reducing substances of the hydrolysate were determined after removal of the sugar with copper sulphate and lime. Since they constituted only 4 to 5 per cent of the total reduction...» CORI and CORI (1930), however, after examining muscles in which they had determined the hydrolyzed glycogen before and after fermentation of the sample, report that »the reducing power in terms of glucose after fermentation of the glycogen hydrolysate was between 0 and 12 mg. per 100 gm of muscle. This small correction of the glycogen values could safely have been omitted without changing the significance of the results.»

SJÖGREN, NORDENSKJÖLD, HOLMGREN and MÖLLERSTRÖM (1938), on examining analyzed samples of liver, found that they contained a reducing substance which they designate as »non-sugar». It is considered by them to represent 10 per cent of the total reduction, and this figure is stated to be fairly constant. SATO (1923) considered he had found a »Restreduktion, die durch nicht-glykogene Substanzen verursacht worden ist». It is, he states, fairly constant in amount and »er beträgt bei Kaninchen in der Leber 0.3 % (als Glukose berechnet), in den Muskeln 0.2 %», and is independent of the content of glycogen. SATO, however, worked with another glycogen determination method, a modification of that of BIERRY-GRUZEWSKA, in which the glycogen is hydrolyzed without washing. His figures therefore are not applicable to the results of the PFLÜGER method.

MAY (1934, a) isolated from a snail a new polysaccharide of galactose, which he terms »galaktogen». In another paper (1934, b), he shows that »galaktogen» occurs also in the mammalian organism under certain conditions in rather large amounts, and that

it is included also in analyses of glycogen. MAY and WEINBRENNER (1938) consider that »galaktogen« is a normal constituent in the mammalian organism, but that in adult males and non-lactating females it occurs merely in insignificant amounts.

As, in my experiments, I always work with adult, non-lactating animals, any »galaktogen« that may possibly occur in connection with them would seem to be a quantité négligeable. According to the author's above reported investigations, any »non-sugar« reducing substances that may possibly occur must be present in such small amounts that they do not affect the results and no regard has therefore been paid to them in the present investigation.

## CHAPTER 2.

### Statistical methods

#### Symbols:

$\bar{x}$  Mean

$\sigma$  Standard deviation =  $\sqrt{\frac{s(x - \bar{x})^2}{n - 1}}$

$\varepsilon$  Standard error of a mean =  $\frac{\sigma}{\sqrt{n}}$

$d$  Difference

$d$  Mean difference

$n$  Number of variates

$T\%$  Standard deviation in per cent of the mean.

#### Methods:

I. For computation of the significance of the differences between two means, derived from relatively small groups of animals, t-analysis (*»STUDENT«* 1908, FISHER 1936, BONNIER and TEDDIN 1940) was adopted.

$$t = \frac{\bar{x} - \bar{y}}{\sqrt{\varepsilon_x^2 + \varepsilon_y^2}}; (n_1 \approx n_2)$$

df degrees of freedom (in t-analysis =  $n_1 + n_2 - 2$ )

P Probability that the groups coincide.

P  $0.05$  = coincidence, corresponding to a difference of less than  $2\sigma$  in large groups.

P  $0.05 - 0.01$  = a probable difference, corresponding to a difference of  $2 - 2.5\sigma$ .

$P_{0.01} - 0.003$  = a highly probable difference, corresponding to a difference of  $2.5 - 3 \sigma$ .

$P_{0.003}$  = a significant difference, corresponding to a difference of more than  $3 \sigma$ .

II. For computation of the significance of differences between the means derived from more than two groups of samples, analysis of variance (FISCHER 1936, BONNIER and TEDDIN 1940) was adopted, the variance ratio ( $F$ ) being calculated according to SNEDECOR (1940).

## Routine of the experiments

As already pointed out, and as will be further explained in the sequel (p. 43), even minor deviations in the experimental conditions may entail marked variations in the amount of the body glycogen deposited. It is thus of the greatest importance that the experimental conditions should, so far as possible, be standardized. I therefore give here a brief review of the general routine of the experiments. Further particulars will be reported in the next section of this work.

Except where otherwise stated, the animals were treated in the following way: —

*Uniform breed.* As has been frequently pointed out, it is of the greatest importance that the experimental animals should be of the same breed. In fact, many investigators have taken their laboratory animals and controls from the same litter. ORTEN and SAYERS (1942) state that they had found a distinct difference between two different breeds of rats. — Owing to lack of accommodation, the author has found it impossible to use a uniform breed throughout, but he has tried to secure the greatest possible uniformity by purchasing almost all the animals from the same breeder. At any rate, all the animals in a series and the corresponding controls came from the same supplier.

The animals were left *at least one week* in the »stable», under uniform conditions, before they were used for experiments, in order to compensate possible differences due to transport and change of environment.

The *diet* for mice consisted of ordinary bread, Gard's »mouse bread», as well as milk and water *ad libitum*. The rats were put on the same diet. The rabbits were fed on hay, oats and

turnips. During the summer, when merely a few experiments were made, the turnips were replaced by fresh green grass.

On these diets the animals grow normally. They are sprightly and lively, show no signs of deficiency, and the fertility is normal.

**Composition of the mouse bread:** 100 kg of milk, 53 kg of crumbs (unspiced), 20 kg of oaten groats, 15 kg of wheat sprouts, 10 kg of lucerne meal, 2.5 kg of minced meat and 40 gm of cod liver oil.

The *temperature* of the environment could not be kept quite constant, but varied between 16° and 21° C. during the periods when the reported experiments were made.

As the *age of the animals* may affect the amount of the glycogen depots, animals of about the same age were always used, their weight being taken as an indication of their age.

**Sex.** Almost all the mice used were female. As regards rats and rabbits, it was found impossible to procure a sufficient number of one sex: for this reason, each series included as a rule an equal number of males and females.

**Controls:** The controls were always examined concurrently with the experimental animals.

**Mode of killing:** Apart from certain special series, all the animals were killed by decapitation with a heavy axe. Immediately afterwards, they received a blow which broke the lumbar cord. The postmortal spasms in the body were thus avoided.

**Dissection for analysis, etc:** As soon as possible after death, a slice of liver is dissected for analysis, is weighed on a torsion balance with a scale of 10 mg, and is plunged into hot KOH within 50 seconds after decapitation. As regards rabbits and rats, a piece of muscle is dissected, weighed and immersed in hot KOH within 90 seconds after decapitation. In rabbits m. triceps s. is regularly used, in rats m. triceps surae and plantaris from one side or both. In mice, the liver is first dissected, weighed and laid in hot KOH. Then the spleen and alimentary canal from the stomach to the rectum are removed. The rest of the animal is cut into four pieces, which are plunged into hot KOH within 90 seconds after decapitation.

**Serial experiments:** All the experiments were serial, and all comparisons are based on the averages from the series.

*Insulin:* A crystalline insulin of the Vitrum brand was used throughout. It contains 22 international units (U) per mg and is dissolved in physiological saline solution. The dosage is always given in U.

*Injection:* The rabbits remain quite quiet during injection. The rats are held with a firm grip over the lower jaw, the body being otherwise free. The mice are left free on the table and the insulin is injected subcutaneously, whilst they are pulled lightly by the tail.

Care is taken not to excite the animals, and they are always treated as gently as possible.

PART II

SURVEY OF FACTORS  
NORMALLY INFLUENCING THE  
GLYCOGEN STORAGE



## The glycogen reserves of the body

The important substance glycogen was first detected and studied by CLAUDE BERNARD. He discovered in 1850 that the liver gave off sugar to the blood. He considered at first that this sugar developed from protein, but in a series of works in the course of the immediately following years he indicated the principal properties of glycogen; in 1855 he presumed that it was a »sorte fécale animale» in the liver, and in 1857 he suggested the method for its production in a pure form. Glycogen has since been of central importance for the study of the metabolism. CLAUDE BERNARD had already indicated the liver and the skeletal muscles as the principal storehouses of the body for glycogen. This view has subsequently been confirmed and accepted, and investigations of variations in the glycogen reserves of the body have, generally speaking, been confined mainly to those two organs. Other organs contain merely minor amounts of glycogen.

Under certain special conditions, however, the adipose tissue may store considerable amounts of glycogen and sometimes it may actually have a glycogen content otherwise found only in the liver. In normal animals the adipose tissue as a rule contains merely insignificant amounts of glycogen. GIERKE (1907) showed that the adipose tissue in guinea-pigs which, after fasting for three days, had been put on abundant diet, was still free from glycogen after 2—3 days, but after 7 days contained considerable amounts of glycogen, which then again decreased. The fact that the adipose tissue in animals which, after fasting for some length of time, had been put on a high carbohydrate diet, during a certain period contained an abundance of glycogen has afterwards been confirmed, for example by ARNDT (1927) on dogs, rabbits and man, HOFFMANN and WERTHEIMER (1927) on dogs, WERT-

HEIMER (1928), RICHTER (1931), EGER and MORGESTERN (1938) and EGER (1942) on rats. That considerable amounts of glycogen may occur in fat is indicated by the fact that ARNDT, under these conditions, found a glycogen content of up to 7.4 %, and EGER up to 6 %, in the adipose tissue. RICHTER moreover ascertained that the administration of insulin had no effect on this storage of glycogen in the adipose tissue. According to SCOZ (1929), considerable amounts of glycogen are stored in the subcutaneous tissue of dogs under similar conditions.

Glycogen thus occurs in large amounts in the adipose tissue only under certain special conditions, lengthy fasting and afterwards an abundant diet for some length of time. These conditions were not provided in my experiments, and my chief interest was therefore devoted to the study of the glycogen variations in liver and muscles. Only in a couple of smaller series on rats was the content of glycogen in the skin also analyzed. In studies on mice the whole body was analyzed, whence the glycogen variations in the adipose tissue are included in the total.

It is generally known that the glycogen reserves are subject to considerable variations. PFLÜGER, as far back as 1902, clearly realized the very marked individual fluctuations, and in recent times attempts have been made to standardize the experimental conditions, in order as far as possible to work with glycogen amounts of the same magnitude. The requirements that are nowadays set up have been clearly formulated by GUEST and RAWSON (1939): »In glycogen determinations large deviations from animal to animal appear to be characteristic. In order to materially reduce this variability a standardization of controls has been attempted. The precautions taken and the results obtained are outlined below:

- 1) An inbred strain of Wistar rats is used.
- 2) Only males are used.
- 3) The age of the animals is restricted to  $100 \pm 4$  days at the time of the sampling.
- 4) A uniform dry pellet diet which contains more than the minimum of all factors necessary for the maintenance of growth and health is fed.

5) An inanition period is established during the interval from 72 to 12 hours preceding the sampling. Food is given *ad lib.* during the final 12 hours, beginning at 10 p. m.

6) The liver and muscle samples are taken between 9:30 and 11:30 a. m.

7) The weight of the dried stomach contents is subtracted from the weight of all the food eaten and all animals in which less than 35 mgm. per gram of body weight has passed the stomach are excluded.

8) Since rats are nocturnally active, a light is left on until 10 p. m. at the time the feeding begins.

9) The temperature of the environment is maintained at 28° C. during the 72 hours preceding the sampling.

10) Anesthesia is by intraperitoneal injection of 0.70 mgm. per kgm body weight, of nembutal. Induction occurs within 4 minutes.»

The question as to the effect which all these different factors may have on the amount of the glycogen reserves is, of course, of the greatest importance for an investigation of this nature. I will therefore give a brief review of the various factors which are considered to affect the amount of the glycogen reserves in normal animals.

## Distribution of glycogen in the glycogen reserves

The distribution of glycogen within the reserves is a problem of the greatest importance if we work with micromethods and let a small piece, or a few pieces, of an organ represent the organ in its entirety. Such procedure is, of course, permissible only if the distribution of glycogen is *uniform* over the whole organ. If the entire organ is subjected to analysis, this problem will, of course, be eliminated.

A. *The glycogen distribution in the liver* has been the subject of much discussion. Thus, for example, LUCHSINGER (1875), SEEGEN and KRATSCHMER (1880), KÜLZ (1886) and CRAMER (1888) considered that the distribution was uniform over the whole liver, whereas v. WITTICH (1875), BARFURTH (1885) and other investigators contended that it varied. GRUBE (1905) considered that the glycogen content was uniform throughout in the actual hepatic parenchyma, but that it varied in different parts of the liver in correspondence with the amount of connective tissue. Among later investigators, SCHÖNDORFF (1903), MACLEOD and PEARCE (1911), PAULESCO (1913), FOLIN, TRIMBLE and NEWMAN (1927), EVANS, TSAI and YOUNG (1931), HOLMGREN (1936) and SJÖGREN, NORDENSKJÖLD, HOLMGREN and MÖLLERSTRÖM (1938) support the view that the distribution is uniform, whereas *e. g.* SCHEIFF (1931) considers that it varies. This question is discussed — after a review of the literature and in relation to the theories regarding the distribution of the portal blood stream in different parts of the liver —, by HENSCHEN (1932), HOLMGREN (1936) and other authors.

*Fig. 9. Table. Comparison between glycogen content of right and left part of the liver in normal rats. Sample of right part of the liver in hot KOH within 50 secs. of decapitation and sample of left part within 90 secs.*

No. of experiments	Glycogen content of right part per cent $\bar{x}$	Glycogen content of left part per cent $\bar{x}$	Mean difference and its standard error $\bar{d} \pm \varepsilon_{\bar{d}}$
12	5.69	5.68	$-0.01 \pm 0.18$

*Own investigations.* In view of the great importance of this problem and the disparity of the results, I have studied this question in experiments on rabbits and rats. As regards rats, the analyses were made in the following manner: — As soon as the untreated animals had been decapitated, the abdomen was opened, and a piece of the right part of the liver was cut off, weighed and plunged into hot KOH, within 50 seconds after decapitation. Immediately afterwards this procedure was repeated with a piece of the left part of the liver and this piece was plunged into hot KOH within 90 seconds after decapitation. According to my investigations, the postmortal glycogenolysis has not yet set in so early after decapitation (cf pp. 47 and 53).

The results of my study of the glycogen distribution in the liver of rats are tabulated in Fig. 9. These investigations show that there is no systematic difference in glycogen content between the two halves of the rat liver.

In my studies of the rabbit liver the procedure was as follows: —

From the right part of the liver a slice of liver was cut out and divided into a number of pieces of suitable size. They were weighed as rapidly as possible and plunged into hot KOH. This procedure was then repeated with a similar piece from the left part of the liver. All the pieces were placed in hot KOH within 5 minutes after decapitation. According to my investigations, the postmortal glycogenolysis in the liver has indeed begun 5 minutes after decapitation, but it has not then yet attained such magnitude that the liver glycogen values statistically

*Fig. 10. Table showing the glycogen percentage of different parts of the liver in normal rabbits. The abdominal wall was cut open immediately after decapitation. A long rod-shaped piece from the right liver lobe was cut into smaller pieces, which were laid in hot KOH. Immediately afterwards the same procedure was repeated with a piece of the left liver lobe. All liver pieces lay in hot KOH within 5 mins. after decapitation.*

Exp.	Right liver lobe		Left liver lobe		Difference in per cent of mean	$d \pm \varepsilon_d$	df	t	P
	Number of samples	Glycogen content per cent	Number of samples	Glycogen content per cent					
A	6	3.06 ± 0.06	6	3.04 ± 0.16	-0.7	-0.02 ± 0.17	10	0.118	>0.9
B	7	2.61 ± 0.08	5	3.02 ± 0.13	+13.4	0.41 ± 0.16	10	2.733	0.02
C	8	11.98 ± 0.41	8	12.89 ± 0.37	+7.3	0.91 ± 0.55	14	1.655	0.2—0.1
D	6	8.72 ± 0.28	6	8.20 ± 0.46	-6.1	-0.52 ± 0.63	10	0.981	0.4—0.3
E	6	0.28 ± 0.02	6	0.22 ± 0.02	-24.0	-0.06 ± 0.03	10	2.000	0.1—0.05
						$\bar{d} \pm \varepsilon_{\bar{d}}$			
						-2.1% ± 6.4			

differ from those obtained on analysis within 50 seconds after decapitation.

Five rabbit livers were analyzed in this way, and the results are tabulated in Fig. 10. As indicated by the t-analysis, a difference between the glycogen content of the two hepatic lobes appeared to be probable in one case. If, however, the mean difference is computed, no systematic difference will be found. The results do not rule out the possibility of some difference in glycogen content between the hepatic lobes, but they argue against the systematic occurrence of such a difference. That the post-mortem glycogenolysis can have no essential bearing on these results is indicated by the fact that sometimes more, sometimes less glycogen is found in the subsequently analyzed piece.

If pieces of liver are taken at random from a series of animals, a serviceable average value should thus be obtained. That this is in fact the case is shown by the analyses of rabbit liver made in connection with the study of the initial postmortem glycogenolysis (see p. 50). They were made on pieces of liver taken at random from different parts, at as short intervals as possible. The results are tabulated in Fig. 19. The table shows that, when liver samples are taken serially from animals, comparable glycogen values will be obtained, which in this connection is the essential.

*B. The distribution of glycogen in the skeletal muscles.* Also as regards the skeletal muscles, we must as a rule content ourselves with analyzing a small sample and letting it represent the whole musculature. Only small animals, such as mice and possibly rats, can be studied and analyzed in toto. It is therefore of fundamental importance to ascertain whether the different muscles of the body have the same glycogen content, or whether there are regular variations between different muscles.

As regards mammals, NASSE (1877) considers himself to have found considerable variations, likewise CRAMER (1888), MOSCATI (1907) and CHOI (1928). On the other hand, ELIAS and SCHUBERT (1918), FOLIN, TRIMBLE and NEWMAN (1927), LONG (1928) as well as EVANS, TSAI and YOUNG (1931) find good correspondence between different muscles. BEST, HOET and MARKS

(1926) likewise find a good correspondence between the symmetrical muscles in cats. SAHYUN, SIMMONDS and WORKING (1934), whilst finding a good correspondence between the symmetrical muscles of rats, state that they had observed a higher glycogen content in the muscles of the hindlegs than in those of the fore legs. MASAYMA and RIESSER (1931) find a higher glycogen content in the white than in the red muscles in rabbits.

A number of studies on the glycogen content in different muscles of birds have been published, but seem to be devoid of interest in this connection.

Several investigators, such as CORKILL (1930) and GOLDBLATT (1930), have simply taken a single muscle as representative of the whole skeletal musculature.

The author's own investigations were made on rabbit muscles. They were firstly in the nature of double determinations of the glycogen content in different parts of the same muscle, from which two pieces were taken for analysis as soon as possible after decapitation. — This material will be discussed in connection with the question of the initial postmortem glycogenolysis, and the results are tabulated in Fig. 20.

Secondly, in a series of normal rabbits, different muscles were dissected as soon as possible after decapitation and analyzed for their glycogen content. The first muscle was plunged into hot KOH within 50 seconds after decapitation and the last within 5 minutes. As shown by the studies on postmortem glycogenolysis reported further on, no change in the glycogen content of the muscles, as compared with the sample taken after the lapse of 50 seconds, occurs during the first 15 minutes after decapitation.

The results of these investigations are tabulated in Fig. 11. This table shows that there is no difference in glycogen content in the different muscles examined. In Fig. 12 the same values are tabulated in the form of averages for the glycogen content of different muscles in the individual animals. Here we find a marked variation and apparently in several cases statistically significant differences. If this material, however, is subjected to an analysis

*Fig. 11. Table. Estimation of glycogen content of different muscles in the rabbit. The muscles were prepared as rapidly as possible and laid in hot KOH within 50 secs. to 5 mins. of decapitation. The muscles were prepared in the order given in the table.*

M u s c l e	No. of examined muscles	Glycogen content per cent $\bar{X} \pm \varepsilon_{\bar{X}}$
M. triceps sin . . . . .	13	0.25 $\pm$ 0.03
M. triceps dx . . . . .	13	0.28 $\pm$ 0.04
M. quadriceps sin . . . . .	13	0.26 $\pm$ 0.08
M. quadriceps dx . . . . .	13	0.31 $\pm$ 0.03
M. erector trunci . . . . .	13	0.35 $\pm$ 0.05
M. semitendineus (red) . . . . .	9	0.38 $\pm$ 0.06

of variancee (see Fig. 13), it will be found that these variations are due to chance.

Thus, in studies of this nature we seem to be warranted in reckoning with a uniform distribution of the glycogen not only in the same muscle, but also within the entire skeletal museulature, so that a glycogen value from a single muscle may be considered to be representative of the whole system.

*Fig. 12. Table. Results shown in Fig. 11 grouped together for every separate animal and given as the average glycogen content of its muscles. Where only five muscles were examined, m. semitendineus was always the one omitted.*

No. of muscles	Glycogen content per cent $\bar{X} \pm \varepsilon_{\bar{X}}$	No. of muscles	Glycogen content per cent $\bar{X} \pm \varepsilon_{\bar{X}}$
5	0.21 $\pm$ 0.01	6	0.37 $\pm$ 0.04
6	0.25 $\pm$ 0.02	5	0.40 $\pm$ 0.03
6	0.55 $\pm$ 0.04	5	0.31 $\pm$ 0.01
6	0.30 $\pm$ 0.04	5	0.13 $\pm$ 0.02
6	0.36 $\pm$ 0.04	5	0.19 $\pm$ 0.04
6	0.27 $\pm$ 0.03	5	0.15 $\pm$ 0.01
6	0.24 $\pm$ 0.04		

*Fig. 13. Table showing the results of an analysis of variance of the results tabulated in Fig. 12.*

Cause of variation	Degrees of freedom	Square	Mean square
Between the groups . . . . .	12	0.8551	0.0711
Within the groups . . . . .	59	0.3418	0.0058
Total . . . . . . . . . . .	71	1.1949	
$F = \frac{0.0711}{0.0058} = 1.226$			
$P = > 0.2$			

### Summary:

No systematic difference in the distribution of glycogen in different parts of the glycogen reserves could be shown. Even if rather marked differences between different parts of the same animal may occur, it will doubtless be possible, by analyzing part of the glycogen reserve from series of animals, to obtain an average value which is representative of the reserve as a whole. This applies both to the liver and to the skeletal muscles.

## Mode of killing and postmortal glycogenolysis

**I. Mode of killing.** Generally speaking, two methods of killing the animals are reported in the literature: Decapitation and narcosis.

SAHYUN and LUCK (1929), in their glycogen analyses, take pieces of muscle from the hind legs of the animals. They consider that the sciatic nerve should be cut off, in order to avoid spasms and a consequent loss of glycogen. ANDERSON and MACLEOD (1930) state that the postmortal spasms are largely accountable for the individual differences in the content of muscle glycogen. MACLEOD and PEARCE (1911) state that these spasms can be avoided by decapitating the animals in the upper thoracic region.

EVANS, TSAI and YOUNG (1931) consider that more liver glycogen is lost if the animals are decapitated than if they are narcotized with ether. CORI (1931), however, recommends large doses of amyta! intravenously for cats and rabbits, intraperitoneally for rats. GUEST and RAWSON (1939) recommend nembutal intraperitoneally for rats. NUTTER (1941) considers that too low muscle glycogen values are obtained if the animal is decapitated, as glycogen is then consumed in the muscle spasms. She recommends, instead, that amyta! should be given intravenously and states that in this way she finds less deviation in the values.

The question regarding the mode of death is closely connected with that of the effect of narcosis on the glycogen stores, which is also of the greatest importance for all experiments of long duration under narcosis. It will, of course, always be necessary to show that the changes observed in these experiments are not due to the narcosis.

CROFTAN (1908) states that during an ether narcosis of 10—15 minutes he can perform laparotomy without affecting the liver glycogen. EVANS, TSAI and YOUNG (1931) likewise state that a short ether narcosis for cats after a fast of 16—48 hours does not affect the liver glycogen. MACLEOD and PEARCE (1911), on the other hand, consider that the irregularities in the deposition of glycogen in the different parts of the liver are accentuated under ether narcosis.

It is a generally known fact that the blood sugar rises under ether narcosis (See, for example, EPSTEIN, REIS and BRANOWER, 1916, EPSTEIN and ASCHNER, 1916). CHROMETZKA and BEUTMAN (1940) state: »Selbst bei vorsichtiger Ätherapplikation kann es bei entsprechender Narkosedauer zu Blutzuckersteigerungen höchstens Ausmasses kommen. Bei kurzdauernder Äthernarkose zum Zweck der Narkosevertiefung ist der Blutzuckereffekt geringer und flüchtiger». Most authors also find that ether narcosis tends to reduce the liver glycogen. LAUBER (1938) states that the decrease in liver glycogen is approximately proportional to the duration of the narcosis, whilst LAUBER and BERSIN (1939), as the effect of an hour's narcosis on a rabbit, found »...dass der Glykogengehalt der Leber während der Narkose um etwa 50 % sinkt». DAOUD and GOHAR (1933—1934) consider that ether narcosis reduces the liver glycogen by 40—50 %. EVANS, TSAI and YOUNG (1931) consider that amytal narcosis after a few hours markedly lowers the content of liver glycogen. About 50 % are lost in two hours. HINES, LEESE and BARER (1928), with a differently planned investigation, had previously arrived at the same result. EDLUND (1942) states: »Eine mässig starke einmalige Dosis Narkotal verursacht keine Veränderung des Glykogen — und Fettgehaltes der Leber».

STEINMETZER and SWOBODA (1928), summing up, state: »Alle untersuchten Narkotika bewirkten eine Hyperglykämie... Die durch die Narkotica erzeugte Hyperglykämie wird als eine Enthemmungserscheinung auf das im Hirnstamm gelegene Zuckzentrum aufgefasst.» EISLER and HEMPRICH (1932) report that »bei Luminalgaben in nicht tödlichen Mengen wurde stets eine leichte Erniedrigung beobachtet», namely in the blood sugar. HRUBETZ and BLACKBERG (1938), after testing a number of

barbiturates, arrived at the result that »with each of the barbiturates studied there was a marked depression in the glycogenolytic power of the liver...»

CORI (1931) writes: »...one must be aware of the fact that all anaesthetics so far available, including amyntal, have a depressive influence on the glycogen formation in the liver».

EVANS, TSAI and YOUNG (1931) discuss what bearing the loss of body heat during narcosis may have on the decrease of the liver glycogen, and TACHI and TAKAI (1926) consider it to be of fundamental importance.

Reports regarding the effect of narcosis on the muscle glycogen are found but sparsely in the literature. MOSCATI (1907) categorically states »... jedenfalls kann der Chloroform, das die Muskeln zur Er schlaffung bringt, im Gegensatz zu manchen anderen chemischen Stoffen bei nicht zu langer Dauer der Einwirkung kein Einfluss auf den Glykogengehalt zugeschrieben werden.» SCHENK (1923), having studied the effect of chloroform narcosis on dogs, states: »Der Glykogenvorrat des Muskels ist am Ende der Narkose und in den folgenden Tagen herabgesetzt». He supposes that chloroform causes cellular damage which tends to retard the glycogen synthesis. DAOUD and GOHAR (1933—1934) found the muscle glycogen unchanged after ether narcosis, whereas the liver glycogen was reduced by 40—50 %. HINES, LEESE and BARER (1928), having studied the effect of glucose infusion on dogs under amyntal narcosis, state: »It was found that approximately the same increase in muscle glycogen had occurred in animals with and without amyntal anesthesia».

It thus appears from the literature that all narcotics of considerable duration result in a reduction of the liver glycogen. Reports regarding the effect of a short narcosis are more sparse, but the general view seems to be that their effect is less or none. The reports regarding the reaction of the muscle glycogen are not very enlightening.

In my *own investigations* I adopted only a very brief narcosis. For my analyses of the initial postmortem glycogenolysis (see below), I found it necessary to anesthetize the animals, so that they could be immediately dissected. Thus, the effect of the narcotic lasted merely a few minutes. In the table Fig. 14 mice which

*Fig. 14. Table. The effect of avertin anesthesia on the glycogen content of the body. Males. Food ad lib. Animals killed at 3 p. m. The animals were given 5 mgm. avertin by intraperitoneal injection. The anesthesia was induced in 30—60 secs., when the animals were immediately decapitated and dissected in the usual way. The control animals were decapitated unanesthetized and dissected in the same way.*

	Controls $\bar{X} \pm \epsilon_{\bar{X}}$	Avertin animals $\bar{X} \pm \epsilon_{\bar{X}}$	$d \pm \epsilon_d$	t	df	P
Liver glycogen per cent	$1.84 \pm 0.29$ (n = 11)	$1.68 \pm 0.32$ (n = 11)	$0.16 \pm 0.43$	0.872	20	0.8—0.7
Body glycogen per cent	$0.118 \pm 0.014$ (n = 11)	$0.114 \pm 0.020$ (n = 11)	$0.004 \pm 0.024$	0.167	20	0.9—0.8
Mgm. glycogen per 10 gm. weight minus alim. canal and spleen.	$21.9 \pm 2.8$ (n = 11)	$20.4 \pm 3.8$ (n = 11)	$1.5 \pm 4.7$	0.819	20	0.8—0.7

have been decapitated and dissected in the usual way are compared with mice which have been decapitated and dissected under amytal narcosis. They first received an injection of avertin intraperitoneally and, when after about 30 seconds to 1 minute it had induced narcosis, the animal was decapitated. As shown by the said table, there is no difference whatsoever between these two groups in regard to liver or muscle glycogen.

Many investigators have in fact adopted this procedure without preliminary testing and have taken their samples of organs under a brief anesthesia of some kind. On the other hand, especially in the case of large experimental series, this procedure entails no advantages as compared with simple decapitation.

**II. Postmortal glycogenolysis** is a phenomenon of fundamental importance for an investigation such as this. It is, of course, essential to make the analyses as soon after death as possible, so that the values are not changed by postmortal glycogenolysis. Whether this is technically practicable or not depends on (1) how soon the postmortal glycogenolysis sets in and (2) how soon the samples can be prepared. From earlier authors we

find statements to the effect that it had taken them about 30 minutes to one hour to prepare the organ in question before laying it in hot KOH. In accordance with my own technique, the sample for liver analysis lies in hot KOH within 50 seconds, and the muscle sample within 90 seconds, after decapitation. Greater rapidity can scarcely be attained in practice.

The second above-mentioned question as to how soon the postmortal glycogenolysis sets in now remains to be discussed.

A. *The postmortal glycogenolysis in the liver.* The reports on this subject in the literature show great divergence. CLAUDE BERNARD (1855), in his earliest experiments, ascertained that the liver develops sugar postmortally and in course of time he discovered that this sugar is formed by the decomposition of glycogen. Many earlier authors, such as BOEHM and HOFFMAN (1880), KÜLZ (1881), and GARNIER and LAMBERT (1897), state that they had observed a slow glycogenolysis in the liver. LUBARSCH (1906) mentions »eine ziemlich rasche Zersetzung des Glykogens sobald das Leben der Zellen unterbrochen ist«. MEIXNER (1911) found that within one hour post mortem 16—54 % of the liver glycogen disappears in rabbits and 100 % in guinea-pigs. MACLEOD and PEARCE (1911) showed that the postmortal glycogenolysis in the liver sets in within the first 20 minutes after death, and that it has its greatest intensity in the course of the first hour. Afterwards, they state, it continues at a fairly constant rate for several hours. EVANS, TSAI and YOUNG (1931) state that no glycogen is lost during the first 40 seconds. Setting out from this value, they find a gradual diminution, so that after 8 minutes the liver contains only 60 per cent. of the initial glycogen value. BOBBIT and DEUEL (1940) find that the glycogenolysis in liver substance kept outside the body at 37° C is »much slower than generally believed«. They state that the intensity of the glycogenolysis varies in different animals, being most marked in rats and diminishing in the following order: guinea-pig, rabbit, dog. On the basis of their relatively small investigations, these authors report that the liver glycogen in one hour diminished by 31 % in rats and 13 % in rabbits. BOMSKOW and v. KAULLA (1942) emphasize the importance of rapid dissec-

*Fig. 15. Table. The post-mortem glycogenolysis. The glycogen content of liver pieces taken from the same liver lobe of normal rabbits at different times after decapitation. Each value is given as percentage of the glycogen content of the first sample, which lay in hot KOH within 40 secs. of decapitation. The livers were then placed in a damp chamber at room temperature.*

Time after decapitation	No. of animals	Remaining glycogen per cent of first sample $\bar{x} \pm \epsilon_{\bar{x}}$
40 secs.	15	100 $\pm$ 0
5 min.	14	94 $\pm$ 4
15 "	15	84 $\pm$ 5
60 "	15	71 $\pm$ 5
150 "	14	64 $\pm$ 5
240 "	15	60 $\pm$ 5

tion in order to avoid the complicating effect of the postmortal glycogenolysis. They consider it to be sufficient if the sample of the tissue is placed in the hot potash lye within 2½ minutes.

In short, the reports in the literature regarding the postmortal glycogenolysis in the liver are by no means in good agreement so far as relates to its intensity and the time at which it sets in.

My own investigations were made on rabbits and rats. The first sample of liver was taken immediately after decapitation, and the piece lay in hot KOH within 40 seconds. The rabbit liver

*Fig. 16. Table. The post-mortem glycogenolysis. The glycogen content of liver pieces of normal rats at different times after decapitation. Each value given as percentage of the glycogen content of the first sample, which lay in hot KOH within 40 secs. of decapitation. The livers were then left in situ at room temperature.*

Time after decapitation	No. of animals	Remaining glycogen in per cent of first sample $\bar{x} \pm \epsilon_{\bar{x}}$
40 secs.	19	100 $\pm$ 0
5 min.	16	86 $\pm$ 8
15 "	17	62 $\pm$ 4
75 "	17	42 $\pm$ 3

Fig. 17. Diagram showing the post-mortem glycogenolysis in the liver.

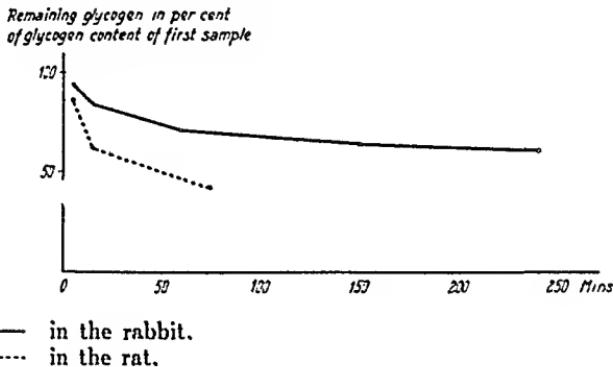
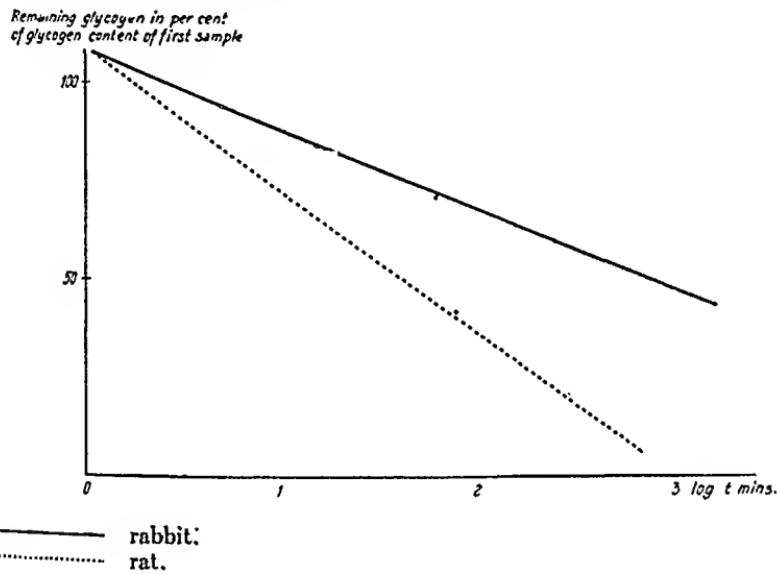


Fig. 18. Diagram showing the post-mortem glycogenolysis in the liver.

Lines of regression constructed according to the method of least squares.



was then dissected and placed in a moist chamber at room temperature. New samples were taken at intervals (see Fig. 15). The studies on rats were made in the same manner, except that the liver, after the first sample, was left *in situ* at room temperature. In order to prevent evaporation, the abdomen was closed in the interval between the samplings. The results are tabulated in Fig. 16.

The course of the glycogenolysis can be read from the diagrams in Figs. 17 and 18. It will be seen from Fig. 18, where the line of regressions has been constructed according to the method of least squares, that the postmortal glycogenolysis in rat liver starts 1.8 minutes, and in rabbit liver 2.6 minutes, after decapitation. This, of course, is correct only on the assumption that the glycogen content in the first sample is really representative of the liver glycogen at the moment of decapitation, *i. e.* that no glycogenolysis has occurred during the 40 seconds it takes to prepare the first sample. This question will be discussed under the section on the initial postmortal glycogenolysis (p. 53).

The glycogenolysis sets in earlier and has greater intensity in the rat than in the rabbit. BOBBIT and DEUEL's unproved statement has thus been verified. The diagrams on Figs. 17 and 18 also show that the postmortal glycogenolysis, as already pointed out by MACLEOD and PEARCE, is most rapid in the course of the first hour, indeed, during the first half-hour after death.

In order to verify the figures thus found for the time at which the glycogenolysis sets in, tests were also made with double liver samples as soon as possible after decapitation. The first sample lay in hot KOH within 40—50 seconds, the second within 30—40 additional seconds, after decapitation. Both of them had thus been placed in hot KOH before the time at which the glycogenolysis, according to the above data, should set in. The result of these analyses is tabulated in Fig. 19. According to these investiga-

*Fig. 19. Table. Glycogen content of two successive liver samples, taken as soon as possible after decapitation. The first one was laid in hot KOH within 40—50 secs. of decapitation, the second 30—40 secs. later.*

Animal species	No. of animals	Glycogen content per cent		$\bar{d} \pm \varepsilon_{\bar{d}}$
		Sample I	Sample II	
Rat. . . . .	21	2.18	2.21	$0.03 \pm 0.03$
Rabbit ser. I . . . .	16	7.84	7.77	$-0.07 \pm 0.07$
, , II . . . .	13	7.15	6.99	$-0.15 \pm 0.16$
, , III . . . .	21	2.18	2.21	$0.03 \pm 0.03$

tions, there is no systematic difference between the glycogen content in the first and second liver sample, that is to say, no loss of glycogen had occurred in the interval between the two tests.

B. *The postmortal glycogenolysis in muscles.* This question has received less attention than has been given to the same process in the liver.

BOEHM (1880), unlike a number of earlier authors, found no diminution of the muscle glycogen within 2 hours. KÜLZ (1881) states that the process is very slow. CRAMER (1888) finds a substantial decrease in the course of 4 hours and stresses the importance of rapid dissection. MOSCATI (1907), working with operative material from man, considers that »der Anfangswert für den Glykogen sinkt nur langsam ab, eine Stunde nach dem Absterben verändern sich nur die dritte oder zweite Dezimale«. MCKAY (1928) considers that glycogenolysis in intact muscle proceeds slowly. CORI (1931) writes: »In the first few seconds after stunning the animal, glycogenolysis is very rapid, but as the lactic acid content of the muscle rises to higher and higher values, glycogenolysis is slowed down more and more and eventually comes to a standstill. Since the sampling of the muscle generally falls into the latter period, the error introduced by the loss of glycogen is apt to be relatively constant in the different experiments. For some investigations it may therefore not be absolutely necessary to determine true glycogen values, but it would seem preferable to avoid the possibility of error and hence of wrong conclusions.« CORI (1930) recommends that, after vivisection under amyta barbital narcosis, the muscle should at once be laid in hot KOH.

This view as to the very rapid initial postmortal glycogenolysis is based mainly on the studies of DAVENPORT and DAVENPORT (1928), who show that the only way of obtaining low lactate acid values in muscles is immediate freezing of the whole intact muscle. Even if the freezing is not deferred for more than 2–6 minutes after death, these authors find a substantial increase in the content of lactate acid, which is considered by them to be produced by the decomposition of glycogen. These authors attach

*Fig. 20. Table.* *M. triceps sin. of rabbit was dissected immediately after decapitation. It was divided into two portions, which in rapid succession were weighed and placed in hot KOH. The first one lay in hot KOH within 50 secs. of decapitation, the second within additional 20—30 secs.*

	Sample I $\bar{X} \pm \varepsilon_{\bar{X}}$	Sample II $\bar{X} \pm \varepsilon_{\bar{X}}$	$\bar{d} \pm \varepsilon_{\bar{d}}$	n
Glycogen content per cent	0.39	0.41	0.02 ± 0.03	56

very great importance to muscle spasms and the manipulations during dissection as causes of the rapid decomposition of the glycogen. GOLDBLATT (1933), on the other hand, considers that uniform and reliable values for the muscle glycogen can be obtained on analysis for up to one hour after death. ANDERSON and MACLEOD (1930) state that »no change occurs in the amount of glycogen in intact mammalian muscle as a result of standing at room temperature for 1 hour after death». SIMPSON and MACLEOD (1927), on the other hand, arrive at the result that »in mammalian muscle which is frozen and ground up in liquid air immediately after its removal from a living animal (decapitated cat) and then allowed to stand at room temperature, glycogen almost entirely disappears within 20—30 minutes». The disparity in the results may be explained by the fact that glycogenolysis in injured tissue is much rapider than in intact tissue (EVANS, TSAI and YOUNG, 1931, and others).

Thus, the reports in the literature are divergent also in regard to the postmortal glycogenolysis in the muscles. As this question is of the greatest importance for the present study, a control investigation of it seems necessary.

My own investigations comprise two series. In view of CORI's above reported views, based on the studies of DAVENPORT and DAVENPORT, a series of investigations were first made on *musculus triceps brach. sin.* of normal rabbits. The muscle was dissected as soon as possible after decapitation, and was divided into two pieces, which were weighed and laid in hot KOH, the first piece

*Fig. 21. M. triceps sin. of rabbit was dissected immediately after decapitation and divided in two. The one was immediately weighed and placed in hot KOH within 50 secs. of decapitation, the other kept for 15 mins in a damp chamber at room temperature before being placed in hot KOH.*

Time after decapitation	No. of animals	Muscle glycogen content per cent $\bar{X} \pm \varepsilon_{\bar{X}}$
50 secs . . . . .	13	0.28 $\pm$ 0.05
15 mins . . . . .	13	0.28 $\pm$ 0.04

within 40—50 seconds after decapitation and the second within 30—40 additional seconds. The results of these tests are tabulated in Fig. 20. We see from this table that the mean difference between the glycogen content in the two pieces is less than its standard error. The correspondence in regard to the amount of glycogen is thus good and no systematic deviation is found.

In the second series the muscle was dissected in the same way and divided into two pieces. One of them was immediately weighed and laid in hot KOH within 50 seconds after the decapitation. The other piece was kept in a moist chamber at room temperature for 15 minutes before it was weighed and laid in the hot KOH. The results of the analyses are tabulated in Fig. 21, which shows that the amount of glycogen is the same in both pieces. Thus, no glycogenolysis had occurred during the interval between the first test and the second. According to CORI, however, the glycogenolysis is most rapid during the first seconds after death, after which equilibrium is attained. According to the reports of DAVENPORT and DAVENPORT, longer intervals of time must have been involved than those in question here (see the immediately following section).

C. *The initial postmortal glycogenolysis.* The entire above reasoning is valid only under the assumption that the amount of glycogen in the sample plunged into hot KOH within 50 seconds after decapitation is really the same as in the organ at the moment

*Fig. 22. Table. Normal mice. Females. Food ad lib. The mice were alternately decapitated and dissected in the usual way and killed under avertin anesthesia, the alimentary canal removed and the remainder cut into pieces, which were dropped directly into hot KOH.*

	Controls $\bar{x} \pm \varepsilon_{\bar{x}}$	Avertin animals $\bar{x} \pm \varepsilon_{\bar{x}}$	$d \pm \varepsilon_d$	t	df	P
Total glycogen expressed in per cent of body-weight minus alimentary canal and spleen.	$0.184 \pm 0.027$ (n = 9)	$0.186 \pm 0.014$ (n = 9)	$0.002 \pm 0.031$	0.065	16	>0.9

of decapitation. In order to throw light on this question, a number of additional experiments were made.

These experiments were made on mice narcotized with avertin. They received an injection of 5 mg of avertin intraperitoneally, on which dose they lose consciousness in about 30 seconds to 1 minute. According to my previously reported investigations on the effect of short avertin narcosis on the glycogen stores of the body (see Fig. 14 p. 46) an avertin narcosis of this duration has no effect whatever on the glycogen contents in the organs.

It has been presumed that the glycogen left in the organs after a fast of 24 hours is retained with great tenacity, whereas the glycogen in non-fasting animals is very easily attacked and rapidly disintegrated. For this reason, the initial glycogenolysis was studied on animals with and without 24 hours fasting.

1. On a series of normal mice under avertin narcosis the alimentary canal was quickly cut out, whereupon the remainder of the animal was cut into four pieces, which were directly plunged into hot KOH. This animal series was compared with concurrent series of animals which were decapitated and dissected in the ordinary way without narcosis. The results are tabulated in Fig. 22. The glycogen values show very good correspondence. In these tests without fasting, the liver contains large amounts of glycogen.

*Fig. 23. Table. Normal mice. Males. 20 hours fasting. Killed at 8 a. m. The controls were decapitated and dissected in the usual manner. The animals of the test series were anesthetized with avertin and cut alive into pieces which were dropped directly into hot KOH. Earlier experiments showed that the alimentary canal after this period of fasting does not contain any polysaccharides which might affect the result.*

	Controls $\bar{x} \pm \varepsilon_{\bar{x}}$	Avertin animals $\bar{x} \pm \varepsilon_{\bar{x}}$
Glycogen content in <i>per cent.</i>	$0.042 \pm 0.003$ (n = 11)	$0.042 \pm 0.002$ (n = 11)

2. After 24 hours fasting, the liver in mice contains merely insignificant amounts of glycogen. A glycogen analysis made in the same way as in the preceding series will thus be in the first place representative of the rest of the body, notably of the muscles. In preliminary investigations it was moreover shown that the intestinal lumen in mice after 20 hours fasting does not contain any polysaccharides, whence also the intestine under these conditions may be included in the analysis. In this series the entire avertin-narcotized animal was cut into four pieces, which were directly plunged into hot KOH. This series was compared with the concurrently studied series of animals, where they were decapitated and dissected in the usual manner without narcosis. The results are tabulated in Fig. 23. The table shows that there is no difference in the glycogen content between the two series. As a supplementation of this series, an experiment was made in which the animals were decapitated after 24 hours fasting and then immediately cut into pieces, which were plunged into hot KOH, in which they lay within 12 seconds after decapitation. Also these animals were compared with a series of animals which had been decapitated and dissected in the usual way without narcosis. The results, which are tabulated in Fig. 24, show good correspondence between the glycogen values of the two series.

3. In different quarters stress has been laid on the importance of rapidly interrupting the post-mortem glycogenolysis, and attempts have been made to attain this in various ways. For example, the dissected organs have been immediately frozen with

Fig. 24. Table. The initial post-mortem glycogenolysis. Normal mice. Males. 24 hrs. fasting. Killed at 5 p. m. The animals of one series immediately after decapitation were cut into pieces and dropped into hot KOH within 12 secs. The controls dissected in the usual manner.

	Controls $\bar{x} \pm \epsilon_{\bar{x}}$	Animals cut to pieces within 12 secs $\bar{x} \pm \epsilon_{\bar{x}}$	$d \pm \epsilon_d$	t	df	P
Glycogen con- tent in per cent	$0.041 \pm 0.004$ (n = 10)	$0.044 \pm 0.007$ (n = 10)	$0.003 \pm 0.008$	0.875	18	0.8-0.7

carbonic acid snow or in liquid air, (SAHYUN and LUCK, 1929), which, however, did not show other glycogen values than if the pieces had been laid direct in hot KOH.

The most rapid way of interrupting the glycogenolysis in small animals seems to be to kill them by casting them into liquid air. A mouse in that case will be frozen in 10 to 15 seconds. In my experiments I used mice which after 27 hours fasting, were cast into liquid air. When they had frozen they were cut in pieces and laid in hot KOH. In that way all processes in the organs had been interrupted as rapidly and effectively as possible. These animals were compared with the control series in which the animals, after fasting for 27 hours, had been decapitated and immediately after decapitation had been cut into four pieces, which were plunged into hot KOH. As the liver in mice after this fasting period contained merely a very minute amount of glycogen, the analysis results obtained are practically representative of muscle glycogen. The are tabulated in Fig. 25. This table shows that there is no marked difference between the two animal series. The probability that a difference exists is  $P = 0.05$ . This result seems to indicate the desirability of further investigations. It should be noted, however, that it is the animals killed by being thrown into liquid air that show a lower amount of glycogen than those treated in the usual way. Consequently, these tests have already shown that freezing with liquid air at any rate does not involve any advantage as compared with decapitation and dissection in the usual way.

*Fig. 25. Table. Comparison between animals killed by being thrown into liquid air and animals killed by decapitation, cut into pieces and dropped into hot KOH. The frozen animals were sectioned and placed in hot KOH. Mice. 27 hrs. fasting.*

Treatment	No. of animals	Weight gm	Glycogen content in per cent
Liquid air . . . . .	16	13.5	0.071 ± 0.003
Decapitation . . . . .	16	13.5	0.079 ± 0.002
			$d = 0.008 \pm 0.004$
			$t = 2.000$
			$df = 30$
			$P = 0.05$

A matter which must be taken into account here is the question whether the hot potash lye penetrates into a tissue as rapidly as it thaws, as otherwise there is every prospect of obtaining in the interval a rapid glycogenolysis. It has in fact been shown, for example, by SIMPSON and MACLEOD (1927) and EVANS, TSAI and YOUNG (1931) that the rate of the glycogenolysis is greatly increased in damaged tissue.

### Summary.

These investigations show that no difference in the amount of glycogen can be observed in mice treated as rapidly as is possible and in mice treated in the standard way adopted throughout in this investigation. As the fasting animals in the above reported investigations have a very low content of glycogen in the liver, the glycogen values are practically representative of the muscles, and consequently there is no change in the glycogen content of the muscles during the first 90 seconds after decapitation. According to previously reported studies of glycogenolysis in rabbit muscles, there was no difference in glycogen content in the organs analyzed 50 seconds after decapitation and those which had not been analyzed until 15 minutes later. CORI's view regarding an extremely rapid initial post-mortal glycogenolysis has thus not been verified.

These experiments thus bear out the previous supposition that the samples of liver and muscles lying in hot KOH within 90 seconds after decapitation may be regarded, with respect to glycogen content, as representative of the organ at the moment of decapitation.

In this regard, it is of no consequence whether the animals are rich in glycogen (being treated without preceding fasting) or are poor in glycogen after fasting.

All these investigations were made at about the same time of day, namely in the morning. It is conceivable that the post-mortal glycogenolysis might vary at different times in the 24 hours, just as the glycogenetic and glycogenolytic action of the liver seems to be subject to cyclic variations in the course of the day. This question has not been investigated, as I was merely concerned with the practical question of ascertaining the amount of the glycogenolysis and when it sets in after decapitation at the time of the day when I conducted my experiments.

## The bearing of the animals' age and sex on the amount of the glycogen depots

A. Sex. In the earlier literature we find no reports on the bearing of sex on the amount of the glycogen depots. On the other hand, since the middle of the nineteen-thirties this problem has been attacked in regard to the liver by a number of investigators.

GREISHEIMER and JOHNSON (1930) could not show any difference between the sexes with respect to the amount of liver glycogen in rats. DEUEL, GULICK, GRUNEWALD and CUTLER (1934) arrived at the same result as regards rats and guineapigs, whereas GULICK, SAMUELS and DEUEL (1934) found somewhat lower liver-glycogen values in females than in males when the animals after 48 hours fasting received 5 mg. of glucose per cm<sup>2</sup> body surface and were afterwards fasted for another 48 hours before the analysis. BLATHERWICK, BRODSHAW, CULLIMORE, EWING and LARSON (1936) state, in regard to the glycogen content in the liver and muscles in their normal material of rats: »The sex difference in the glycogen content of their tissues was clearly apparent». In regard to the liver, the males had undoubtedly higher glycogen values. As regards the muscles, however, the difference was less convincing. DEUEL, HALLMAN and MURRAY (1937) show higher liver glycogen in male than in female rats and DEUEL, BUTTS, HALLMAN, MURRAY and BLUNDEN (1937) state that »the level of liver glycogen in the females averaged only 60 per cent of that in the liver of the males». In the young animals, however, they found no sex differences. DEUEL, HALLMAN, MURRAY and SAMUELS (1937) consider that the deposition of glycogen in the liver of rats, on administration of glucose *per os* after fasting, is larger in males than in females. This, they state,

is not due to the difference in resorption, as the females under these conditions resorb much more rapidly than the males. The difference is considered by them to be due to greater oxidation in the females. MERTEN (1939), »Die weiblichen Tiere (rat) wiesen stets einen niedrigeren Glykogengehalt der Leber auf«, GRAYMAN (1941), confirms the view of DEUEL *et alios*.

As regards the rabbit, SJÖGREN, NORDENSKJÖLD, HOLMGREN and MÖLLERSTRÖM (1938) have found that the males have a higher liver glycogen content than the females. Moreover, the females lose their glycogen more rapidly than the males during fasting.

NEUFELD and COLLIP (1941) could not show any sex difference in the liver glycogen content in mice.

A matter of the greatest importance for investigations of this nature is the variation in the series. DAOUD and GOHAR (1933) found that male rats were more sensitive than the females to factors which might affect the content of liver glycogen. NEUFELD, SCOGGAN and STEWART (1940) as well as NEUFELD and COLLIP (1941) state that they have obtained more regular figures for females than for males, but that the average value is the same.

*Own investigations.* For my studies on rabbits it was impossible to arrange sufficiently large series with animals of one sex only. I have therefore tried, instead, to have an equal number of males and females in each experimental series, in order to obtain comparable values even if there should be any sex difference. From the animal material in the normal series males and females were picked out just for this special question and were compared with one another. The results are tabulated in Figs. 26 and 27. These tables show no difference in the amounts of the glycogen depots in the males and females.

For mice and rats experimental series were arranged for direct determination of possible sex differences. The results are tabulated in Figs. 28 to 29. As will be seen from these tables no sex difference could be shown in regard to the liver and muscle glycogen in my material of non-fasting rabbits, rats and mice. On the other hand, the standard deviation in the series is somewhat less for females than for males, just as DAOUD and GOHAR, NEUFELD, SCOGGAN and STEWART as well as NEUFELD and COLLIP had

*Fig. 26. Table showing the storage of glycogen in male and female rabbits and rats. Normal rabbits and rats. Decapitated at 5—8 p. m. Normal rats, 2 hrs. fasting, decapitated at 11 a. m.*

Animal species	Sex	No. of animals	Body weight gm $\bar{X} \pm \epsilon_{\bar{X}}$	Liver weight gm $\bar{X} \pm \epsilon_{\bar{X}}$	Muscle glycogen per cent $\bar{X} \pm \epsilon_{\bar{X}}$
Rats	♀	9	124 ± 5	5.0 ± 0.3	2.42 ± 0.41
	♂	11	115 ± 5	4.6 ± 0.2	2.65 ± 0.51
Rabbits	♀	24	1644 ± 76	64.1 ± 3.8	7.74 ± 0.80
	♂	21	1593 ± 79	66.0 ± 4.1	8.88 ± 0.82

*Fig. 27. Table. The glycogen storage in normal rabbits and rats. The difference between males and females tabulated in Fig. 26.*

Liver glycogen				Muscle glycogen			
$d \pm \epsilon_d$	t	df	P	$d \pm \epsilon_d$	t	df	P
Rabbits . . .	-1.14 ± 1.14	1.000	48	0.4 — 0.3	0.03 ± 0.04	0.750	48.
Rats . . .	-0.23 ± 0.65	0.354	18	0.8 — 0.7	-0.07 ± 0.04	1.750	18 0.1 — 0.05

*Fig. 28. Table. The storage of glycogen in normal male and female mice. Food ad lib.*

		Glycogen content in mgm per 10 gm of body weight minus alim. canal and spleen					
		No. of ani- mals	Body weight gm $\bar{X} \pm \varepsilon_{\bar{X}}$	Liver weight mgm $\bar{X} \pm \varepsilon_{\bar{X}}$	Alimentary canal spleen mgm $\bar{X} \pm \varepsilon_{\bar{X}}$	Liver glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$	Body glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$
Group A Decap. at 8—9 a.m.	♀	28	22.5 ± 0.8	1.135 ± 44	3.611 ± 127	1.94 ± 0.18	0.071 ± 0.006 (n = 27)
	♂	25	22.8 ± 0.5	1.067 ± 29	3.311 ± 85	1.66 ± 0.22 (n = 24)	0.072 ± 0.004 (n = 24)
Group B Decap at 5 p.m.	♀	14	20.7 ± 1.0	1.115 ± 82	3.471 ± 279	0.96 ± 0.16	0.088 ± 0.006 0.099 ± 0.007
	♂	16	21.2 ± 0.9	1.016 ± 82	3.341 ± 149	0.51 ± 0.17	12.3 ± 1.8

*Fig. 28. Table. The difference between males and females tabulated in Fig. 28.*

		Body glycogen				Glycogen content in mgm per 10 gm of body weight minus alim. canal + spleen			
		d ± ε <sub>d</sub>	df	t	P	d ± ε <sub>d</sub>	df	t	P
Group A	0.28 ± 0.29	51	0.966	0.4 — 0.3	-0.001 ± 0.006	50	0.166	0.9 — 0.8	3.1 ± 2.0
	0.45 ± 0.23	27	1.957	0.1 — 0.05	0.011 ± 0.009	28	1.222	0.8 — 0.2	2.1 ± 1.8

already pointed out. I have therefore used only females in the principal series for mice.

In the literature it will be found that most authors, if they had reflected at all about this question, had used only males, without further investigation of the conditions. The reason is stated to be that in this way they believe that they escape from the possible effect of the female sexual cycle. BOKELMANN and DIECKMANN (1930) as well as BOKELMANN, DIECKMANN, KAUFMANN and SCHERINGER (1931) state that they could show a connection between the glycogen reserves of the animal and sexual cycle, but their differences are not of such a magnitude as to be statistically significant. No connection between these factors has hitherto been demonstrated.

**B. Age.** Since CLAUDE BERNARD (1859) examined fetus livers and was unable to show any liver glycogen until the second half of the embryonal life, a number of investigations of fetal liver and muscle glycogen have been made. The earlier studies were made with more or less defective technique and therefore had scarcely any decisive importance for the question when glycogen first begins to be deposited and in what quantities it then occurs. BARFURTH (1885) states that, in examinations of fetuses, he had not observed any glycogen in the liver, but had found it in several other organs of rabbit, sheep and guineapig in different stages of development. In homo fetuses which had died during delivery SALOMON (1874) found an abundance of glycogen, whereas MARCHAND (1885) was unable to observe any. Nor was the latter able to find any glycogen in a lamb aged a few months, 15 minutes after death.

PFLÜGER (1903), who worked with a better technique, observed liver glycogen during the first half of the development of the fetus, likewise SUNDBERG (1924) with a histological technique. Similar reports are found in ADAMOFF (1905). Livini (1927) detected traces of glycogen in the liver of a homo fetus 18 mm in length.

Even if there is some divergence of opinion as to when the liver first begins to deposit glycogen, it is generally agreed that the glycogen content of the liver is at first low, even lower than

that of the muscles. (PFLÜGER 1903, GIERKE 1905, LOCKHEAD and CRAMER 1906, LIVINI 1927, STIEVE and KAPS 1937, and others.) The amount of liver glycogen is considered to be low at first, but increases considerably towards the end of fetal life (LOCKHEAD and CRAMER, LIVINI, STIEVE and KAPS).

The muscle glycogen content, on the other hand, is high in the fetus already at an early stage (GIERKE, PFLÜGER, SUNDBERG).

Thus, to judge by the literature, the liver seems at first to contain merely small amounts of glycogen, at any rate not more than e. g. the skeletal muscles. It is not until just before birth that the glycogen content rises. In other words it is not until this stage that the liver begins to act as a store for glycogen.

According to STIEVE and KAPS, the glycogen content of the liver during the first weeks of extra-uterine life lies at about the same level as at birth. HAX (1927), who worked with puppies considered that they had a liver rather poor in glycogen at birth and that the glycogen content increased during the suckling period. JONEN (1924), who studied new-born dogs, found an average glycogen content of 5 % in the liver and 0.6 % in the muscles.

DEUEL, BUTTS, HALLMAN, MURRAY and BLUNDEN (1937), studied the liver glycogen in rats of different ages. All the animals were put on the same diet and were killed at 8—10 a. m. during a short period in the spring. They state: »The level of glycogen present in the liver of unfasted rats is highest at 39—40 days, at which time it exceeds 8 per cent. From this level it gradually drops to a value of about 4 per cent, which is found in male rats 75 days of age. Approximately the same values were noted in rats 19—24 months old. There was no sexual difference noted in the liver glycogen of rats 26—29 days of age nor in old rats (17 to 24 months). In the other groups the level of liver glycogen in the females is constantly lower than that of the males. The liver glycogen reached a minimum value in the females which were three months old.»

HEYMAN and MODIC (1939) studied rats aged 8—11 days, 10—12 weeks and 2—3 years. All the animals were killed at the same time of the day after a fast of 48 hours. »The liver glycogen values for rats 8 to 11 days old is one-third that for rats in the

two older groups. During the first 6 weeks of post natal life this value increases steadily, the average values for the animals 6 weeks old being 5.5 gm per 100 gm of fresh liver, which is only slightly higher than the average value for the full-grown animals... The increase in content of glycogen in the liver of baby rats takes place mainly during the nursing period... The muscle glycogen values in baby rats are certainly not lower than those in the two older groups, if they differ at all... Fasting for 1 and 2 days diminishes the content of glycogen in liver and muscle of rats in all three groups with the same ease. This does not support the hypothesis that the liver retains its glycogen depots with greater tenacity during infancy than during later periods of life. BOMSKOW and v. KAULLA (1942), after experiments on guineapigs, likewise stress the bearing of age on the amount of the glycogen depots. Those authors also find a rising content of muscle glycogen with advancing age. The series in their work are, however, too small for their figures to be quite convincing, in view of our knowledge of the considerable individual variations.

To judge by the literature, the investigators seem more or less consciously to have evaded the source of error involved in possible differences in the glycogen content with varying age. Without any discussion and without giving reasons, they have taken animals of approximately the same size, *i. e.* age, which indeed seems quite natural. In correspondence with this, GUEST and RAWSON (1939) recommend very narrow age limits for animals used for studies of the glycogen depots. If the animals' weight is adopted as an indicator of their age, however, regard must be paid to the fact that *e. g.* female rats of fertile age grow more slowly than males (DONALDSON, 1908).

*Own experiments.* The liver and muscle glycogen values for rabbits, mice and rats of different ages were compared. The rabbit series for these age studies were composed from different control series, it having been found, as explained in the preceding section, that sex does not play any part in the glycogen content of the rabbit. As regards mice and rats special series were arranged for this purpose. The animals in the different age groups were treated in exactly the same way and were studied at the same time. The procedure was the same as in the principal series. The results

*Fig. 30. Table showing the storage of glycogen in rats and rabbits at different ages.*

Animal	No.	Body weight gm $\bar{X} \pm \epsilon_{\bar{X}}$	Liver weight gm $\bar{X} \pm \epsilon_{\bar{X}}$	Liver glycogen per cent $\bar{X} \pm \epsilon_{\bar{X}}$			Muscle glycogen per cent $\bar{X} \pm \epsilon_{\bar{X}}$
				d	$\pm \epsilon_d$	P	
Rats	12	153 ± 6	6.0 ± 0.3	1.97 ± 0.27	—	—	—
	12	40 ± 1	1.8 ± 0.1	1.94 ± 0.41	—	—	
Rabbits	26	1879 ± 51	76.1 ± 2.8	9.88 ± 0.58	0.87 ± 0.03	—	—
	16	1214 ± 45	46.1 ± 2.9	5.72 ± 0.76	0.31 ± 0.03	—	

*Fig. 31. Table showing the storage of glycogen in rats and rabbits at different ages. The differences between the animals tabulated in Fig. 30.*

	Liver glycogen				Muscle glycogen			
	d	$\pm \epsilon_d$	df	t	d	$\pm \epsilon_d$	df	t
Rats	0.03 ± 0.49	22	0.061	> 0.9	—	—	—	—
	3.66 ± 0.93	40	3.935	< 0.001	0.06 ± 0.04	40	1.500	0.2 ± 0.1

Fig. 32. Table showing the storage of glycogen in mice at different ages.

	No. of animals	Body weight g/m <sup>1</sup>	Liver weight mg/m <sup>1</sup>	Mincillary canal and spleen weight		Liver glycogen per cent $\bar{X} \pm E_{\bar{X}}$	Body glycogen per cent $\bar{X} \pm E_{\bar{X}}$	Glycogen content in mgs per 10 gm of body weight minus alum. canal + spleen $\bar{X} \pm E_{\bar{X}}$	
				$\bar{X}$	$E_{\bar{X}}$			$\bar{X}$	$E_{\bar{X}}$
Series I	16	23.6 ± 0.7	1.035 ± 0.5	2.893 ± 0.75	3.68 ± 0.23	0.114 ± 0.010	29.7 ± 1.9	32.7 ± 5.4	20.4 ± 9.2
	14	11.6 ± 0.3	0.621 ± 0.17	2.116 ± 0.55	2.71 ± 0.12	0.113 ± 0.016	2.71 ± 0.12		
Series II	16	23.4 ± 1.3	1.162 ± 0.52	3.624 ± 1.95	1.66 ± 0.26	0.073 ± 0.009	(n = 14)	13.4 ± 1.6	43.3 ± 4.2
	16	10.3 ± 0.3	0.521 ± 0.29	1.958 ± 1.03	1.73 ± 0.12	0.045 ± 0.012	(n = 15)		
Series III	10	21.5 ± 1.2	1.239 ± 0.18	—	1.98 ± 0.54	0.118 ± 0.015	31.6 ± 3.9	27.0 ± 2.7	0.526 ± 0.5
	10	12.1 ± 0.1	0.726 ± 0.49	—	3.21 ± 0.11	0.115 ± 0.009	—		

Fig. 33. Table showing the storage of glycogen in mice at different ages. The differences between the estimates tabulated in Fig. 32.

	Liver glycogen				Body glycogen				Glycogen content in mgs per 10 gm of body weight minus alum. canal + spleen			
	$d \pm E_d$	df	t	P	$d \pm E_d$	df	t	P	$d \pm E_d$	df	t	P
Mice I	0.37 ± 0.48	28	0.771	0.5 — 0.1	0.005 ± 0.019	28	0.263	0.8 — 0.7	-3.0 ± 5.7	28	0.526	0.7 — 0.6
Mice II	0.07 ± 0.28	29	0.250	0.9 — 0.8	0.019 ± 0.014	23	0.711	0.5 — 0.4	2.0 ± 2.7	27	0.711	0.5 — 0.4
Mice III	1.74 ± 0.73	18	2.381	0.95 — 0.92	0.003 ± 0.017	18	0.176	0.9 — 0.8	8.7 ± 5.7	18	1.526	0.2 — 0.1

are tabulated in Figs. 30—33. As will be seen from these tables, the two rat series show a very good confirmity in the content both of liver glycogen and muscle glycogen. As regards the rabbits, on the other hand, there is a statistically significant difference ( $P = < 0.001$ ) between younger and older animals, in that the younger show a considerably lower liver glycogen content than the older ones. As for the muscle glycogen no such difference could be observed, and the two series show a good correspondence. As regards mice, the bearing of age on the glycogen depots was investigated in three different experiments. In the two first, older and younger animals show a very good correspondence in regard to the liver glycogen content, whereas in the third series the younger animals were found to have a smaller amount of liver glycogen than the older ones ( $P = 0.05—0.02$ ). As such a difference occurs only in one group and the statistical probability is not definite, it seems unlikely that this is a systematic difference. It seems more probable that some external unknown factor has come into play. As regards the glycogen content in the body a very good correspondence was always found between the older and younger animals, as also for the total amount of glycogen per weight unit.

It may seem curious that age should have a bearing on the liver glycogen content in rabbits, but not in mice and rats. This may possibly be due to the fact that the differences in age were not comparable. To judge by the reports in the literature on the influence of age on the glycogen depots, even if the data are somewhat disparate, it may be inferred that the depots are generally considered to be somewhat larger in older animals than in very young ones.

### Summary.

Some investigators state that in rats, guineapigs and rabbits they have ascertained that the glycogen reserves of the males are larger than those of the females. As regards mice such differences could not be found. In my investigations such sex differences, under the existing experimental conditions, could not be observed in rabbits, rats and mice. On the other hand, the

standard deviation is somewhat less for females than for males. On his account, only females were used for the principal experiments on mice. For purely technical reasons, it was impossible to procure a sufficient number of animals of one sex for the studies on rats and rabbits. I tried, instead, to secure the same number of animals of each sex in the series which were to be compared.

The more extensive investigations reported in the literature indicate that older animals have a larger amount of liver glycogen than very young ones. In my experiments I observed such an age difference in rabbits, but not in mice and rats. All the investigations reported in the sequel were made, so far as possible, with animals of the same age. This was at any rate the case with the serial experimental animals and the corresponding controls.

## The bearing of the diet and the length of the fasting period on the glycogen depots

### I. The effect of the diet.

It is obvious that the composition of the diet must have a considerable bearing on the magnitude of the glycogen reserves, and attention has long been directed to this matter. The earlier experiments were chiefly intended to ascertain how far the glycogen content could be forced up. Thus SCHÖNDORFF (1903) states that in dogs on a meat diet high in carbohydrates the liver glycogen can be forced up to 18.7 per cent and the muscle glycogen to 3.7 per cent. PFLÜGER (1907) put dogs on a lengthy fast and then gave them a monotonous fat, protein or carbohydrate diet. He writes: »Als Ergebnis dieser Untersuchungen darf mit grösster Wahrscheinlichkeit behauptet werden, dass die Leber bei vollkommener Entziehung der Nahrung bis zum Hungertode fortfährt, Glykogen zu bilden. Wird der Leber als Nahrung in überschüssiger Menge entweder nur Fett oder nur Eiweiss zugeführt, so hört die Glykogenbildung auf oder wird auf ein Minimum heradgedrückt. Wird aber der Leber als Nahrung in überschüssiger Menge ausschliesslich Traubenzucker zugeführt, so nimmt die Glykogenbildung in aussärgewöhnlich starkem Maasse zu, wie ja längst bewiesen ist.» JUNKERSDORF (1921) confirms SCHÖNDORFF's results.

In regard to rabbits LIEBIG (1940) states that the liver glycogen is reduced if the animals are put on oats or turnip diet, and that it is normal if they are given chiefly carrots.

OSBORNE and MENDEL (1924) kept rats on a carbohydrate-free diet for several months. The animals grew well. On analysis of

the whole animal a glycogen content of 0.09 per cent was found as compared with 0.12 per cent in animals on normal diet, GREISHEIMER and JOHNSON (1929 and 1930 a) report a definite increase of the liver glycogen in rats put on a diet rich in sucrose and a definite decrease if the diet is rich in fats or casein. GREISHEIMER and JOHNSON (1930 b) write: »Feeding 60 per cent of the total calories in the form of corn starch gave a liver glycogen which did not differ significantly from that on the balanced diet. With 60 per cent of the calorific value in the form of sucrose or lard, a significantly higher liver glycogen was found, while 60 per cent of casein gave a significant decrease. The muscle glycogen content on any of the test diets did not differ significantly from that on the balanced diet.» SAHYUN, SIMMONDS and WORKING (1934) examined different diets and write: »Glycogen content of the muscles of the rat under the conditions of the experiments tends to vary directly with the percentage of carbohydrate in the diet, being over 50 per cent greater in the animals on a high carbohydrate diet than in animals on a high protein diet.» MIRSKI, ROSENBAUM, STEIN and WERTHEIMER (1938) find a decrease of the liver glycogen if a large part of the calories in the diet are supplied in the form of casein or meat. The muscle glycogen on the other hand, was not affected. STEIN, TUERKISCHER and WERTHEIMER (1939) compare experimental animals on a diet the calories of which were supplied as to 34 per cent by butter, 34 per cent by margarine, 26 per cent by casein and 6 per cent by starch, with normal animals the caloric requirements of which are covered as to 70 per cent by carbohydrates. They find that the controls have nearly twice as high a liver glycogen as the experimental animals. HOLMGREN (1944) studies rats put on a diet of bread or boiled beef. Both groups receive a full supply of vitamins and salts. »Die Leberglykogenwerte sind bei Kh-Tieren höher als bei Fleischtieren. Die Differenz, 1.80 % ± 0.54, ist statistisch sicher.»

As regards guineapigs, BOMSKOW and v. KAULLA (1942) state: »Bei der Wechsel von Winterkost (Heu, Rüben und Brot) auf Sommerkost (Gras und Brot) sowohl eine Erhöhung der Leberglykogenwerte wie auch eine Erhöhung der Herzmuskelglykogenwerte, vor allem aber eine Erhöhung der Skelettmuskelglykogenwerte.»

As regards mice, MIRSKI, ROSENBAUM, STEIN and WERTHEIMER (1938) state that the animals put on a wheat diet have twice as high a content of liver glycogen as animals kept on a meat diet.

KAUNITZ and SELZER (1937) find a lower liver glycogen content in rats who had been put on vegetable salt-free diet than in rats who had been put on a meat diet rich in salt. CRABTREE and LONGWELL (1936) state that 9 % sodium chloride in the food increases the liver glycogen in rats, whereas 6 % or deficiency of salt does not result in any deviation from the normal. GALLI and RAFFO (1939) state that intravenous injections of common salt tend to increase the fixation of glycogen in the liver. KOBORI (1926) studied the effect on the liver glycogen of potassium and calcium salts in the food and found that the glycogen content was highest on a high calcium diet and lowest on a low potassium diet. »Doch scheint der Einfluss kein erheblicher zu sein.« The muscle glycogen was found to be unaffected.

As regards the question of the sensitivity of animals to insulin on different diets, much work has been devoted to studying the effect of alkalizing and acidifying diets. Also the effect of such diets on the glycogen depots has been studied, but the results are not uniform. Thus, for example, STEIN, TUERKISCHER and WERTHEIMER (1939) state that acidotic diet entails a rise in the liver glycogen content in the winter, but not in the summer, whereas alkalosis results in normal values. GOLDBLATT (1927) gives rats on normal diet some sodium bicarbonate in the milk, and then finds that the glycogen almost entirely disappears both from the liver and from the muscles. Much value can scarcely be attached to most of the experiments that have been made on this subject. As a rule, like many other experiments in diet, they are not arranged in such a way that the animals can be regarded as »normal and healthy». It is often remarked *en passant*, in a foot note or the like, that a large number of the experimental animals had died, or that they appeared to be ill. Under such conditions one must obviously expect great variations in the glycogen reserves.

The effect of vitamins on the carbohydrate metabolism is a question which has attracted great interest. Their effect on the

glycogen reserves has also been studied. These investigations, however, in many cases are badly planned and the experimental material insufficient, for which reasons the results are often vague and selfcontradictory.

*Vitamin A*, supplied in excess to rats on a normal diet, according to ABELIN (1935) increases the muscle glycogen. He considers that this rise is entailed by the well-known antagonistic effect of vitamin A to thyroxin, which tends to reduce the muscle glycogen. BAUEREISEN (1938) considers that vitamin A in excess produces an increased fixation of glycogen in the liver. It is largest when sugar is concurrently supplied. A lack of vitamin A entails a reduction of the glycogen reserves.

*Vitamin B* has been the subject of a whole series of investigations. FUNK (1914) considered that this vitamin was of importance for the carbohydrate metabolism. In  $B_1$ -avitaminotic pigeons BICKEL and COLLAZO (1923) found a deficiency of glycogen, whereas ABDERHALDEN and WERTHEIMER (1932 and 1934) found the liver of  $B_1$ -avitaminotic pigeons richer in glycogen than that of normal pigeons, and this glycogen rise was considered by them to be directly proportional to the degree of the avitaminosis. This richness of glycogen is confirmed, among others, by TONUTTI and WALLRAFF (1938). In rats, FORNAROLLI and BONI (1940) find a reduction of the muscle glycogen after a period on  $B_1$ -free diet, concurrently with the appearance of other symptoms. According to HERMANN (1939), the liver glycogen content in rats during the first stage on  $B_1$ -free diet shows a rise, but afterwards returns to the normal value, and falls below it in the final stage. TONUTTI and WALLRAFF (1938) state that they have found in rats a reduction of the liver glycogen content in cases of  $B_1$ -avitaminosis. EDLUND and HOLMGREN (1941) came to the same results. The supply of vitamin  $B_1$  in excess, according to LAJOS (1936), produces an increased tendency towards glycogen fixation. According to EDLUND and HOLMGREN (1941) and EDLUND (1942), no definite change in the liver glycogen content could be found after the supply of vitamin  $B_1$  in excess.

*Vitamin C*: PALLADIN (1924) »Beim Skorbut verschwindet das Glykogen aus der Leber und am Ende des Skorbuts war das Glykogen überhaupt nicht nachweisbar« (guineapig). Low liver

glycogen content in scorbutic guineapigs was noted also by ALTENBURGER (1936) and SHIMAMURA (1938).

Several authors have worked with an increased supply of vitamin C and have studied the effect on the liver glycogen. HERMANN (1938) finds a marked increase, MORELLI and D'AMBROSIO (1938), TERADA (1939), FICHERA (1940) and others confirm this, whereas TCHERKES and ROSENFELD (1941) state that a supply of vitamin C in excess results in a reduction on the liver glycogen, and BORELL and HOLMGREN (1945) find no change.

*Vitamin D:* PINCUSSEN (1932) supposes that vitamin D affects the carbohydrate metabolism.

## II. The effect of fasting.

That fasting tends to lower the magnitude of the glycogen reserves is a matter on which there has long been general agreement, but investigators are disagreed regarding the amount of this glycogen decrease.

A) *The liver.* The earlier investigators as a rule work with very long fasting periods. LUCHSINGER (1875) states that the liver in rabbits is free from glycogen after a fast of 6—14 days. ALDEHOFF (1889), on the other hand, states that the liver of the rabbit still contains considerable amounts of glycogen after 6 days fasting and that of the cat after a fast of 14 days. PFLÜGER (1902), in studies on dogs after 28 days fasting, found a liver glycogen content of 4.8 %. He emphasizes, however, the immense individual variations. In 1907 he stated »dass die Leber bei vollkommener Entziehung der Nahrung bis zum Hungertode fortfährt, Glykogen zu bilden«. ISHIMORI (1913) finds a marked decrease of the liver glycogen after 1—5 days fasting. JUNKERSDORF (1921) in studies on monkeys still found 3.12 grams of glycogen in the liver after 23 days fasting. LIEBIG (1940) finds a substantial decrease in the liver glycogen after a fast of 6—7 days.

These long periods of fasting naturally entail profound changes in the function of the liver cells and therefore are devoid of interest in this connection. Later investigators worked with

shorter fasting periods as a means of making the glycogen content in the liver more uniform in serial experiments with the object of inducing changes in the liver glycogen content by different means.

FISHER and LACKEY (1925): »The liver is the first organ to lose its glycogen in starvation in both normal and diabetic dog. The loss in the amount of glycogen is rapid at first but the glycogen left after a few days starvation is given up very slowly.»

After studies on rabbits, MARKOWITZ (1925) states: »The inference is very strong from these experiments that starving rabbits are never glycogen free». He found up to 3 % liver glycogen in starving animals. According to SJÖGREN, NORDENSKJÖLD, HOLMGREN and MÖLLERSTRÖM (1938) a substantial reduction of the liver glycogen is found in starving rabbits. The reduction culminates after 48 hours. After 72 hours fasting the liver glycogen content is higher and the values more irregular. The fast of the first 48 hours has no effect on the daily variations in the glycogen content of the liver.

The studies of ÅGREN, WILANDER and JORPES (1931) on mice show that a 10 hours fast entails a marked reduction of the liver glycogen in those animals, but without abolishing the daily variations. NEUFELD, SCOGGAN and STEWART (1940) report a marked reduction of the liver glycogen after 24 hours fasting and »a fair constancy ranging from 50 to 70 mg %. After 48 hours fasting they find less uniformity in the values. NEUFELD and COLLIP (1941) state in regard to mice that the first 5 hours of the fast do not entail any reduction of the liver glycogen. Afterwards the liver glycogen content falls rapidly during the first 14 hours of the fast, but afterwards keeps approximately constant up to 48 hours. These authors were unable to find any difference in the sensitivity of males and females in this respect.

A number of studies of this question have been made on rats. KARCZAG, MACLEOD and ORR (1925) found a marked decrease of the liver glycogen after 24 hours fast. LAWRENCE and McCANE (1931) showed a diminution of the liver glycogen to 0.4—0.3 % after a fast of 24—48 hours. DITTMAR (1933) after studies on rats, states: »Das Glykogen der Leber wird rasch abgebaut, es sinkt am 1. und 2. Hungertag auf ein Minimum». WETZEL, WOLLSCHITT, RUSKA and OESTREICHER (1936) studied rats on normal

diet. They had merely small series of 4 in each group. The rats were always decapitated and analyzed from 9 to 11 a. m. These authors found after

	24 hours fast	0.78 %	liver glycogen		
2×24	»	»	0.77 %	»	»
3×24	»	»	0.88 %	»	»
4×24	»	»	0.93 %	»	»
5×24	»	»	1.03 %	»	»
7×24	»	»	1.35 %	»	»
9×24	»	»	1.12 %	»	»

MIRSKI, ROSENBAUM, STEIN and WERTHEIMER (1938) found a very substantial reduction in the glycogen reserves in starving rats. After 3 days fasting, however, the glycogen values rise again. Similar results were published by BARBOUR, CHAIKOFF, MACLEOD and ORR (1927), who found in rats higher liver glycogen values after 48 hours fasting than after 24 hours. These findings are in complete agreement with the often astonishingly high liver glycogen values reported by earlier authors, after lengthy fasting tests. Unlike these authors LAJOS (1936) found a continuous fall of the liver glycogen in rats during 4 days fasting.

HEYMAN and MODIC (1939), in studies on rats of different ages, found that the liver glycogen content fell with equal rapidity during starvation, irrespective of the animals' age. The lowest value was reached after 6—10 hours.

B. *Skeletal muscles.* As regards the reaction of the muscle glycogen to starving, LUCHSINGER (1875) considered that it disappeared more rapidly than the liver glycogen. In rabbits according to this author, it has completely disappeared after 2 days fasting. ALDEHOFF (1889), on the other hand, found considerable amounts of muscle glycogen in rabbits and cats after a fast of 6 and 12—14 days respectively. ISHIMORI (1913) writes: »Der Glykogengehalt der Muskulatur zeigt in Uebereinstimmung mit den bisherigen Erfahrungen keine auffällige Abhängigkeit von der Nahrungszufuhr«. JUNKERSDORF (1921) states: »dass der Muskel im Hungern sein Glykogen, wenn auch in geringeren

Mengen, viel zäher zurückbehält als die Leber». KARCZAG, MACLEOD and ORR (1925) found in normal rats a reduction of the liver glycogen to 0.27 % after 24 hours fasting, as compared with 0.40 % in the controls without fasting. MARKOWITZ (1926) found that, though starvation tends to lower the muscle and liver glycogen content, it can never make an animal entirely free from glycogen. A combination of starvation, cooling and strychnine spasms can make the liver, heart and skeletal muscles glycogen-free for several hours. HANDOWSKY (1928) states that the muscle contains an undiminished amount of glycogen after 24 hours fasting. LAWRENCE and McCANE (1931) found in rats a decrease in the muscle glycogen from 0.54 % in normal animals to 0.35 % after 24 hours fasting. It then keeps unchanged for the next 24 hours. WESSELKINA (1932) in experiments on cats, found that the muscle glycogen markedly diminished after fasting. LONG and EVANS (1932) found no definite effect on the muscle glycogen of shorter fasting periods than 48 hours. DITTMAR (1933) found in rats, after a fast of up to 7 days, a slow and approximately uniform diminution of the muscle glycogen. ÅGREN, WILANDER and JORPES (1931) found in mice a considerable decrease of the body glycogen after 10 hours fasting. HEYMAN and MODIC (1939) found the lowest liver glycogen values after 6 to 10 hours fasting.

LIEBIG (1940) found in rabbits a decrease of the muscle glycogen to 0.15 % after 6—7 days fasting, as compared with 0.34 % in the normal animals. NUTTER (1941) found a marked decrease in rats after 24 hours fasting.

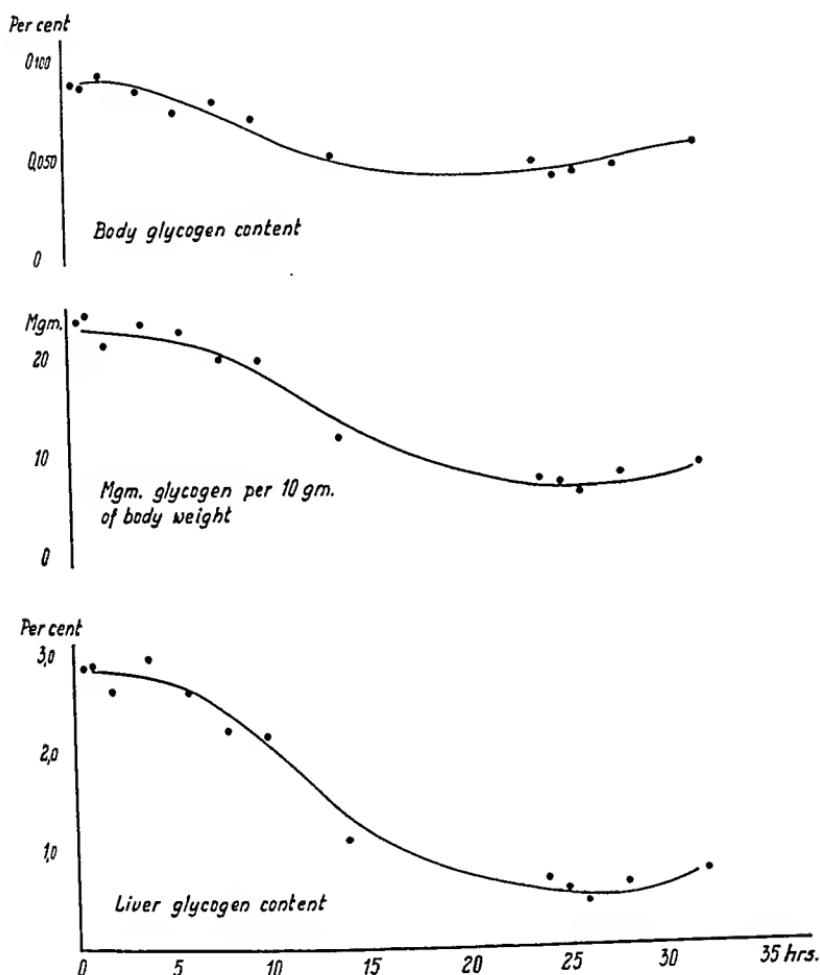
*Own investigations.* For the present investigation the effect of fasting is of the greatest importance, especially for the studies on mice. I have therefore made a tabular summary of my analyses on normal mice with varying fasting periods, all of them decapitated at 8 a. m. The results are tabulated in Fig. 34 and are graphically shown in the diagram Fig. 35:

In complete agreement with the results published by NEUFELD and COLLIP (1941), the liver glycogen content in the animals in my experimental series seems to keep fairly constant during the first five to six hours. It then falls rather rapidly and seems to reach a minimum after about 26 hours fasting, after which it

Fig. 34. Table showing the effect of the length of the fasting period on the glycogen storage in normal mice. Females. Decapitated at 8 a. m.

Time of fasting	No. of animals	Body weight gm $\bar{X} \pm \varepsilon_{\bar{X}}$	Liver weight mgm $\bar{X} \pm \varepsilon_{\bar{X}}$	Alimentary canal and spleen mgm $\bar{X} \pm \varepsilon_{\bar{X}}$		Liver glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$	Body glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$	Glycogen content in mgm per 10 gm of body weight minus alim. $\bar{X} \pm \varepsilon_{\bar{X}}$
				Liver glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$	Body glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$			
25 mins	11	20.7 ± 0.7	963 ± 40	2 448 ± 90	2.86 ± 0.41	0.087 ± 0.005	23.7 ± 2.8	
1 hr	12	19.8 ± 0.6	943 ± 34	2 533 ± 133	2.88 ± 0.18	0.086 ± 0.008	24.1 ± 1.7	
2 hrs	17	21.4 ± 0.3	922 ± 34	2 206 ± 57	2.61 ± 0.26	0.091 ± 0.006	21.2 ± 1.8	
4 "	12	20.9 ± 0.5	954 ± 28	2 420 ± 114	2.95 ± 0.43	0.083 ± 0.008	23.3 ± 2.4	
6 "	21	21.7 ± 0.5	1 102 ± 40	—	2.61 ± 0.22	0.072 ± 0.005	22.5 ± 1.6	
8 "	10	19.6 ± 0.6	955 ± 57	2 573 ± 115	2.21 ± 0.33	0.077 ± 0.004	19.8 ± 2.3	
10 "	21	22.3 ± 0.7	1 178 ± 44	—	2.16 ± 0.14	0.068 ± 0.005	19.5 ± 1.2	
14 "	16	19.8 ± 0.8	1 133 ± 98	—	1.12 ± 0.18	0.049 ± 0.006	11.7 ± 1.4	
24 "	20	18.7 ± 0.2	730 ± 18	—	0.68 ± 0.06	0.044 ± 0.001	7.2 ± 0.3	
25 "	12	18.1 ± 1.1	867 ± 65	2 272 ± 120	0.57 ± 0.06	0.037 ± 0.006	6.9 ± 1.1	
26 "	15	18.3 ± 0.2	715 ± 17	1 765 ± 45	0.44 ± 0.07	0.039 ± 0.003	5.8 ± 0.6	
28 "	12	18.6 ± 0.6	920 ± 38	2 373 ± 87	0.62 ± 0.07	0.042 ± 0.003	7.6 ± 0.6	
32 "	12	17.4 ± 0.4	753 ± 27	2 153 ± 68	0.74 ± 0.06	0.052 ± 0.004	8.5 ± 0.4	

Fig. 35. Diagrams showing the diminished storage of glycogen in mice after different periods of fasting. The diagrams are based on the figures tabulated in Fig. 34.



again rises somewhat. The curves for the body glycogen content and the total amount of glycogen per weight unit show similar variations. For investigations such as the present one, possible changes which set in very rapidly are of the greatest importance, especially as mice have a very rapid metabolism. I have therefore examined the magnitude of the glycogen depots in mice 25 minutes after all food had been removed from the cage. As the

*Fig. 36. Table. The glycogen storage in normal mice. Males. 25 min. fasting. Decapitation at 9 a. m.*

Time of fasting	No. of animals	Body weight $\bar{X} \pm \epsilon_{\bar{X}}$	Liver weight $\bar{m}gm$ $\bar{X} \pm \epsilon_{\bar{X}}$	Alimentary canal and spleen $\bar{m}gm$ $\bar{X} \pm \epsilon_{\bar{X}}$		Liver glycogen per cent $\bar{X} \pm \epsilon_{\bar{X}}$	Body glycogen per cent $\bar{X} \pm \epsilon_{\bar{X}}$	Glycogen content in body weight minus alimentary canal and spleen $\bar{X} \pm \epsilon_{\bar{X}}$
0	10	18.3 ± 0.6	930 ± 90	2317 ± 108		1.21 ± 0.16	0.094 ± 0.007	15.6 ± 1.1
25 mins	9	19.1 ± 0.9	949 ± 80	2549 ± 140		1.03 ± 0.17	0.088 ± 0.008	14.3 ± 1.0
Diff. (d)	—	—	—	—		0.18 ± 0.23	0.006 ± 0.011	1.3 ± 1.6

variations must be very minute, I concurrently studied a series of animals without fasting. The results, which are tabulated in Fig. 36, show very good correspondence for the two series.

### Summary:

As will be seen from the above account, the glycogen reserves and especially the liver glycogen, are very labile, and are affected by several different dietary factors. The experiments which have been made on this subject are often contradictory and the experimental conditions obscure, whence it is not possible to throw clear light on these questions. In experiments where the glycogen reserves are studied it is necessary to work with uniform dietary conditions.

It is a generally recognized and accepted fact that starvation tends to lower the liver glycogen content, and that the reduction after 24—28 hours fasting is very considerable in all animals examined. According to the data in the literature, it seems probable that the liver glycogen content, after reaching a minimum, again rises somewhat. Reports as to how the liver glycogen behaves after a short fast are sparse. Also as regards the muscle glycogen a reduction after fasting is reported, though the figures are more divergent.

According to my studies on mice, there is no change in the glycogen reserves during the first 5—6 hours of the fast. A steady decrease till about 26 hours is then found, and afterwards again a rise. The glycogen content in the rest of the body behaves in the same way.

## Cyclic changes in glycogen content and variations due to temperature

**A. Diurnal variations.** The question regarding variations in the liver glycogen content at different times of the day has been the subject of very animated discussion. It has been concerned not so much with the now accepted view that the liver at different times of the day contains varying amounts of glycogen as with the nature and causes of this variation.

The first observations on the subject were published by FORSGREN in 1927. Two years later he gave a curve for the diurnal changes in the glycogen content of rabbit liver. His investigations were made merely on a small number of rabbits, but they led to a number of deeper studies with different animals.

The first thorough study on the diurnal variations in the glycogen content was published by ÅGREN, WILANDER and JORPES (1931). Summing up their observations on a large material, those authors state: »There are cyclic changes in the glycogen content of the liver of rats, mice and rabbits, which are to a large extent independent of the intake of food. Glycogen accumulates in the liver during the night and disappears again to some extent during the next morning.»

»Similar periodical changes occur, though to a minor extent, in the muscles also.»

These results are confirmed, amongst others, by VAN WEEL (1941).

FORSGREN (1929) published a cyclic curve with two liver glycogen maxima about 4 p. m. and 2 a. m. and a minimum about 10 a. m. This observation has been confirmed by v. EULER and

HOLMQUIST (1934) and afterwards, on a large material, by SJÖGREN, NORDENSKJÖLD, HOLMGREN and MÖLLERSTRÖM (1938).

HOLMQUIST (1931) stated that the curve for the liver glycogen content in the rat ran parallel with that given by FORSGREN (1929) for the rabbit. This statement, however, afterwards proved to be erroneous. According to HOLMGREN'S extensive investigation (1936) on white rats, the liver glycogen maximum for that animal is at about 8 a. m. and the minimum at 4—8 p. m. According to DEUEL, BUTTS, HALLMAN, MURRAY and BLUNDEN (1938), the liver glycogen maximum for the rat is at about 4—8 a. m. and the minimum about twelve hours later.

As regards the guineapig, the liver glycogen content, according to PETRÉN (1939) shows a maximum about 11 a. m.—3 p. m. and a minimum about 5—9 a. m.

According to SECKEL and KATO (1938), the diurnal variations in the liver glycogen content of the rat do not fully manifest themselves before the age of 3 weeks. HOLMGREN (1941) states: »...dass der Leberrhythmus beim Meerschweinchen schon bald nach der Geburt auftritt oder eventuell bei derselben bereits vorhanden ist.«

In regard to the diurnal variations in the glycogen content of the *muscles*, the reports are very scanty. Such variations, however, have been observed by ÅGREN, WILANDER and JORPES.

**B. Seasonal variations.** The question whether the carbohydrate metabolism is subject to seasonal variations has been much discussed, without, however, being definitely decided. As for possible seasonal variations in the liver glycogen content, this question has been studied by many investigators, especially in regard to cold-blooded and hibernating animals. The cold-blooded animals are of minor interest in this connection, and most of the studies on hibernating animals are unsatisfactory, being based on insufficient material.

GÜRBÉR (1895), in regard to rabbits, stated that he had found a liver glycogen content averaging 4.25 % in the summer, as compared with 11.75 % in the winter. He tells us nothing, however, about the diet, time of day, etc. KISSEL (1896), in studies on

rabbits, likewise found a lower glycogen content in the summer than in the winter. On very loose grounds, he rejects the supposition that the variation may be due to a difference in food, considering it to be endogenously involved in the nature of the rabbit organism. FUJII (1924), after two years' study on rabbits at different seasons, states: »The glycogen content of the liver in 151 rabbits was on an average 3.3 %. It underwent a seasonal variation: i. e. was definitely smaller in June and July in both the years» (these being the warmest months). CORI and CORI (1928) state: »Experiences of the past two years have shown that there is a great constancy of the preformed glycogen from year to year as well as at different seasons of the year». It should be noted, however, that this statement refers to rats after a fast of 24—28 hours. BURN and LING (1929) state that the liver glycogen content in rats on a fat diet keeps rather low in the summer, but is higher in the autumn, spring and winter. HIRSCH and VAN PELT (1937), consider themselves to have shown certain seasonal changes in the maximum and minimum glycogen content in the liver. PETRÉN (1939) arrives at similar results. CHROMETZKA and BEUTMAN (1940) state that the first-mentioned author in 1939 had ascertained, as regards hibernating animals, a higher glycogen content in the winter than in the summer. As regards the muscle glycogen, however, he had found the reverse. GOLDBLATT (1929) states: »It has been found in these young animals (rats) that the glycogen content of the liver after 24 hours' starvation is higher in the winter than in the summer».

As regards the content of muscle glycogen, the reports are scantier. FUJII (1924) found no seasonal variations. HANDOWSKY and WESTPHAL (1928), in a study on the carbohydrate content in the skeletal muscles of normal rabbits, state: »Es liessen sich Jahrzeitliche Beeinflussungen nachweisen».

**C. The effect of the temperature of the environment.** All those who engage in the standardization of insulin are well aware that the temperature greatly affects the sensitivity of animals to insulin, and consider it essential that the temperature should be kept constant within rather narrow limits. Similar observations have been made in regard to the glycogen content.

As far back as 1881, KÜLZ observed that the liver glycogen content was reduced by cold. He adopted the rather rough method of immersing rabbits in icy-cold water. LANCZOS (1933 and 1935) made experiments on similar lines, placing mice for 4—5 hours in a tin placed on ice. Though this did not affect the liver glycogen content, it rose when the mice recovered in room temperature. MARKOWITZ (1926) stated that the body glycogen stores were reduced by cold. SILVETTE and BRITTON (1932) that it tended to lower the content of liver glycogen. RAFFERTY and MACLACHLAN (1941) compared the liver glycogen content in rats which had been kept at an outdoor temperature of 35.5°—36.6° C and at 20°—21° C, respectively. They found a higher liver glycogen content in the animals which had been kept at a lower temperature, and the difference was statistically significant. BOMSKOW and v. KAULLA (1942) state that a low outdoor temperature entails a marked reduction in the liver glycogen values of guinea-pigs. At an outdoor temperature of 15° C they found merely 38 % of the liver glycogen content that had been observed at 23° C. The muscle glycogen was unchanged.

As regards from the above, the statements on this question show great variation and systematic studies on sufficiently large material are missing. From the existing scanty reports it seems probable that the liver glycogen content is affected by the outdoor temperature. RAWSON and GUEST (1939) consider that a constant temperature is essential for comparative glycogen investigations.

#### Summary:

Diurnal variations in the liver glycogen content have been shown in most of our usual laboratory animals. This is of the greatest importance for all investigations in which different values for the glycogen content are discussed, and we cannot avoid reckoning with this factor. An investigation which does not take into account such variations is valueless. In order to be in a position to draw any conclusions from differences in glycogen content between experimental animals and controls, it is essential

that they should have been examined at the same time of the day. In the present work the experimental series is always compared with the concurrently examined control series.

The question whether the body glycogen stores are subject to seasonal variations has not been settled. If seasonal variations occur, they may also be due to differences in the outdoor temperature, the composition of the diet, estrus etc. (HANDOWSKY and WESTPHAL). An actual seasonal rhythm has not been shown.

This question is closely connected with that of the effect of temperature. To judge by the scanty data in the literature, it seems probable that variations in the temperature affect the liver glycogen content.

For the present investigation such seasonal changes and temperature variations are of minor importance, seeing that, as already pointed out, the experimental animals and controls are always examined concurrently, and that the temperature in stables and laboratory has been kept between 17° and 22° C.

## Further factors which affect the glycogen content in normal animals

As appears from the above, there are a number of different factors that affect the glycogen content in normal animals, and which can be controlled or avoided in the experiments. There are, however, other factors which entirely elude control and which one must try to neutralize in serial experiments.

The reaction of different experimental animals to insulin under different conditions has been studied very assiduously, and it has been found that all the factors which, according to the above account, affect the glycogen stores also affect the sensitivity of the animals to insulin. A review of the discussion on these questions is given by v. LEDEBUR (1936). According to that author, however, we must also reckon with *e.g.* climatic influences etc., which elude control. LAQUEUR and DE JONGH (1925) speak of »überindividuelle Faktoren«. »Damit soll gesagt sein, es kommen bestimmte Tage, auch Perioden mehrere Tage bis zu einer Woche vor, wo plötzlich sehr starke Reaktionen auftreten können, vielleicht auch mehr Krämpfe als sonst sich bemerkbar machen, und andererseits kommen auch Perioden vor, wo nur besonders schwache Reaktionen beobachtet werden. Man muss dann natürlich an klimatische Faktoren wie Temperatur, Luftdruck, Feuchtigkeit usw. denken.« This fact is well known among all those who work with standardization of insulin preparations. I have myself observed the same phenomenon.

Similarly, the glycogen depots and especially the liver glycogen are subject to uncontrollable variations. Even if every precaution is adopted, one may suddenly find a change in the level of the glycogen content. Such a change is illustrated by the experiments

*Fig. 37. Table. Normal mice. Females. Food ad lib. Decapitated at 8 a. m., one hour after insulin injection. Half of the animals in each group were given 0.008 U. insulin subcutaneously, the other half 0.004 U.*

No. of animals	Date	Body weight gm. $\bar{X} \pm \varepsilon_{\bar{X}}$	Liver weight mgm $\bar{X} \pm \varepsilon_{\bar{X}}$	Glycogen content in mgm per 10 gm of body weight minus alimentary canal and spleen		
				Alimentary canal and spleen mgm $\bar{X} \pm \varepsilon_{\bar{X}}$	Liver glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$	Body glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$
12	8.1.1945	20.3 ± 0.4	931 ± 36	2980 ± 114	3.35 ± 0.39	0.164 ± 0.012
12	2.1.1945	19.9 ± 0.4	843 ± 23	3340 ± 61	1.94 ± 0.25	0.130 ± 0.005
d	—	—	88 ± 43	640 ± 129	1.41 ± 0.46	0.024 ± 0.013
t	—	—	2.047	4.961	3.065	1.846
df	—	—	22	22	22	22
P	—	—	0.1 — 0.05	< 0.001	0.01 — 0.001	0.1 — 0.05
						< 0.001

tabulated in Fig. 37. Both groups of animals were treated in exactly the same way. The experiments were made with an interval of one week. In each group half of the animals received 0.004 and half 0.008 U of insulin subcutaneously without preceding fast. They were decapitated at 8 a. m., one hour after the injection. The difference *between* the two groups is greater than the difference *within* the groups, that is, between the animals which had received different doses. As will be seen from the table there is a statistically significant difference between the two groups in regard to the glycogen content. As regards the body glycogen there is a good correspondence. This is thus apparently the result of the so called »überindividuelle Variation«. If the intestinal weights in the two groups are compared, it will be found that the alimentary canal of the animals richer in glycogen is heavier than that of those poorer in glycogen. The difference, which is  $640 \pm 129$  mg is statistically significant. It is thus conceivable that the difference in the glycogen content may be due to the possibility that the former animals during the night had eaten more or later than the latter and consequently had a higher liver glycogen content. The reason for this possible change in the intake of food, however, eludes control. These sudden variations, which occur also in regard to the glycogen content in normal animals, entail certain consequences in judging the results. Obviously, we cannot immediately compare two otherwise exactly equivalent groups of animals examined at different times. In order that the values may be comparable, the studies must have extended over a considerable length of time, so that the oscillations in different directions can equalize one another.

By comparing an experimental series with a concurrent control series, this error, of course, can be most surely avoided. The possible effects of this kind can be obviated by extending such comparisons over several days. This has been done throughout in the present investigation.



PART III

THE EFFECT OF EXOGENOUS  
INSULIN ON THE GLYCOGEN STORAGE  
OF NORMAL ANIMALS



## CHAPTER 11.

# Survey of the reports in the literature on the effect of insulin on the glycogen storage of normal animals

## A. Different ways of studying the effect of insulin on the glycogen storage.

Attempts have been made in different ways to obtain an idea of the effect of insulin on the glycogen depots in normal and diabetic animals. Here only the studies on normal animals will be dealt with.

1. *In vitro experiments with tissues and tissue slices.* The effect of insulin on the tissues has been studied *in vitro*. See, for example, AHLGREN (1925), v. EULER (1930) and BLASCHKO (1933). Attempts have also been made to study the effect of insulin on the tissue glycogen content *in vitro*. The results have been rather meagre and contradictory.

Thus, for example, COLLAZO, HÄNDL and RUBINO (1924) state »...dass der Glykogenabbau *in vitro* im Leberbrei nicht in nennenswerter Weise beeinflusst wird durch Insulinzusatz.« SECKEL (1938) states: »With a method described in an earlier publication rat liver glycogenolysis as it normally occurs in surviving tissue slices suspended in a buffered salt solution, was shown to be inhibited to a considerable extent by large doses of insulin added *in vitro*.«

»Because of this finding and the results reported in the experimental and clinical literature, the essential action of insulin on the liver is believed to be an inhibition of the glycogenolytic process particularly when the latter is proceeding at a high rate.«

STADIE, LUKENS and ZAPP JR (1939) state: »No effect upon carbohydrate synthesis by liver slices was observed. The carbohydrate synthesis by diabetic cat liver slices was not found increased; here, too, insulin was without effect.» The same authors emphasize this point of view in a publication of 1940: »The new formation of carbohydrate by liver slices of normal or diabetic animals was found to be uninfluenced by the addition of insulin to the equilibrating medium.»

In experiments with rat diaphragms in a Warburg apparatus with a method indicated by GEMILL 1940, GEMILL and HAMMAN (1941) state that they had observed a storage of glycogen in the muscles when insulin was added to the glucose containing suspension medium. The increase in glycogen was small and the reported experiments were few in number, but the results are regular and uniform.

HECHTER, LEVINE and SOSKIN (1941) work with the same methods. They state: »Glycogen deposition in rat diaphragm *in vitro* varies directly with the concentration of glucose in the medium. Insulin catalyzes this process greatly at low sugar concentrations (100 mg %), but very little at high concentrations (400 mg %).»

Summing up, it may be stated that attempts to study the effect of insulin on the glycogen storage *in vitro* have not led to any results as regards the liver, but seem to indicate that insulin under certain conditions may stimulate the storage of muscle glycogen. The absence of effect in experiments *in vitro* is, of course, no proof of a similar condition in experiments on animals, as the metabolism of the tissue is radically changed in experiments *in vitro*.

2. *Perfusion experiments on surviving organs*, for the study of the liver metabolism, were proposed as far back as 1853 by CLAUDE BERNARD. The technique in regard to tortoises was indicated by GRUBE (1907). This animal has the great advantage that both liver lobes can be perfused separately, in which case one of them can serve as a control. In a number of experiments it was shown that, in perfusion tests with the liver of cold-blooded

animals, the building-up of glycogen could be observed. See review by *e.g.* KAPFHAMMER (1925) and NEUBAUER (1925).

It is unnecessary to review here all the different perfusion experiments that have been made on cold-blooded animals. It need only be mentioned that KEPINOV (1938) had found that unpurified insulin preparations increase the glycogenolysis in perfused frog liver, which was not the case with crystalline insulin. This observation is of the greatest importance, especially as most of the perfusion experiments were made at the beginning of the insulin era. At that time investigators worked with more or less impure preparations, which greatly reduces the value of their results. It has been found very difficult to develop perfusion technique so that it could be applied also to the liver of hot-blooded animals, which is very sensitive. The perfusion was at first made with Ringer solution, and the glycogenolysis could not be mastered. The earlier literature on the subject has been reviewed by DE MEYER (1909) and BARRENSCHEEN (1913). The latter, however, states that »bei geeigneten Versuchbedingungen gelingt es, auch an den überlebenden isolierten Warmblüterlebern mit regelmässigkeit Glykogenansatz zu erzielen». LAUFBERGER (1924) states: »Bei Durchspülung der überlebenden Hundeleber trat trotz Insulin eine Vermehrung des Zuckers in der Durchblutungsflüssigkeit ein».

BERNARD (1925) perfuses rat liver with Ringer solution with the addition of glucose in different concentrations. The liver retains sugar proportionally to the glucose concentration in the perfusion liquid. This retention is doubled on the addition of insulin. The retained sugar, however, is not stored in the form of glycogen. »Eine Glykogensynthese wurde mit unserer Methodik nicht erzielt. Das Insulin war ohne Einfluss auf die Geschwindigkeit der Glykogenhydrolyse.»

The results of perfusion experiments on organs from hot-blooded animals are not very encouraging, and CORI (1931) summarizes his experiences as follows: »The results obtained on the isolated mammalian liver are not conclusive... But the chief difficulty in experiments of this kind seems to be that it is practically impossible, even with the best technique available, to keep the mammalian liver outside of the body for any length of time without some serious loss of normal function. For this reason an

effect of insulin which occurs in the intact animal, may fail of demonstration in the isolated organ.»

FIESSINGER *et alios* (1937, 1939 and 1940) state, however, that, with a perfected technique, they had been able to perfuse monkey liver without obtaining any increase in the glucose content of the perfusion liquid, that is to say, without finding any increase in the glycogenolysis in experiments which may have continued for a couple of hours. When they supplied insulin to the perfusion liquid, the liver gave off considerable amounts of glucose to the blood for a time, but soon recovered equilibrium despite the fact that it still contained considerable amounts of glycogen and, on a new supply of insulin could again give off large amounts of glucose. Similar results were reported by BODO and MARKS (1928): »Glycogen storage in the absence of added insulin was observed... Added insulin either stopped further storage or caused a breakdown of glycogen.» The differences reported by those authors were, however, very small. COREY and BRITTON (1941) found, on perfusion of cat liver, that insulin tended to reduce the glycogen content. On the supply of suprarenal extract, they observed an increase.

As regards muscles, the perfusion method has not been of so much interest. Here, instead, investigators could use animals from which the liver had been extirpated for similar studies. (MANN and *co-workers*, BEST, HOET and MARKS, 1926, etc.) In a summary, MANN (1927) states that the supply of insulin increases the consumption of glucose in the muscles. He considers that the liver has no bearing on the development of insulin hypoglycemia, but only on the restoration of the normal blood sugar level. BEST *et alios* likewise consider that insulin acts only via the muscles. When glucose in the blood is in excess, glycogen accumulates in the muscles. When there is a shortage of glucose, the already existing glycogen is retained.

Summing up, it can be said that perfusion experiments with a view to studying the effect of insulin on the glycogen metabolism have been concentrated on experiments with liver. The results have not given any support to the view that insulin stimulates a storage of glycogen in the liver. On the contrary, FIESSINGER and BODO and MARKS indicate that insulin causes an outflow

of glycogen from the liver. Other experiments pointing in the same direction are devoid of conclusiveness in view of evident defects in the technique.

*3. Attempts to draw conclusions regarding the effect on the liver glycogen from the variation of the blood sugar after insulin injection.* MANN and MAGATH (1924) have given a summary of their excellent studies on the part played by the liver in the carbohydrate metabolism, from which it is quite evident that the liver is the only source of the blood sugar. It should be noted, however, that the blood sugar level is dependent on two different factors: the outflow of glucose from the liver and the absorption of glucose in the tissues. For this reason it is impossible to draw any conclusions from the rise or fall of the blood sugar level with regard to an increase or decrease of the glycogenolysis or possible glycogenesis in the liver. We may quite as well be concerned with a decrease or an increase in the peripheral consumption, while the conditions in the liver remain constant. It is still more impossible to draw from the variations in the blood sugar any conclusions regarding changes in the glycogen stores of the liver. The liver glycogen, as we know, is a very labile factor. In regard to its amount, it is at any given moment dependent on the relation between glycogenesis and glycogenolysis, a matter which is often overlooked. Thus, under certain conditions we may find an increase of glycogen concurrently with hyperglycemia (adrenaline effect at certain stages, according to CORI and others).

A rather naïve view — which has been put forward quite seriously —, is that the blood sugar, when it disappears under the effect of insulin, is deposited as liver glycogen and causes a rise in the liver glycogen content. On the contrary, the liver during the whole time when the insulin is acting must, of course, give off glucose to the blood even if the blood sugar level is low, and perhaps particularly just then. Under the action of insulin, the combustion of glucose is generally considered to be stimulated and the amount of glucose in the blood will, of course, suffice merely to maintain the combustion for a few minutes. Fresh glucose must be continuously supplied and it can only come from the liver.

From the above it should be evident that all discussions about the behaviour of the glycogen depots under the action of insulin on the basis solely of blood-sugar studies are meaningless, and I consider it therefore unnecessary to cite any literature on the subject, even if it is very abundant.

*4. Studies on the effect of insulin on the glycogen stores in intact animals.* In studying the effect of insulin on the glycogen stores by direct analyses of organs, investigators have proceeded in two quite different ways. a) Attempts have been made to determine the glycogen content of the organs before and after the supply of insulin and b) the glycogen content in animals after the action of insulin has been determined and compared with the corresponding value for untreated animals.

a) BRENTANO (1939), takes as a basis the following statement by GEELMUYDEN »es ist unmöglich bei Kaninchenversuchen selbst nach der peinlich uniformen Vorbehandlung Kontrolltiere mit einigermassen gleichem Gehalt an Leberglykogen herzustellen. Wenn man ... entscheiden will, ob Insulin die Glykogenspeicherung ... fördert oder hemmt, so sollte diese Entscheidung auf Grund eines Vergleichs zwischen dem Glykogengehalt des Tieres vor und nach der Insulingabe getroffen werden. Diese Idealforderung ist aber in der Praxis kaum zu erfüllen.« BRENTANO continues: »Diese Forderung GEELMUYDENS haben wir — wenn ich nicht irre, als erste — in dem vorliegenden Arbeit erfüllt.« BRENTANO works with normal rabbits. Under light ether anesthesia he extracts a muscle from one of the hind legs and a piece of liver for glycogen analysis. After a couple of days the same procedure is repeated after treatment and supply of insulin. Here, however, there are various possibilities of error, including the so-called »überindividuelle« variation (LAQUEUR and DE JONGH).

Another possible procedure is, in the course of the experiment, to take samples from the liver and muscles and in this way continuously to follow the glycogen variation. Most investigators who have tried this procedure have had their laboratory animals under narcosis. In this way, however, they exposed themselves to the complicating action on the glycogen which may be attributed

directly or indirectly to the narcosis. CORI (1931) states: »When this is done under anesthesia, one must be aware of the fact that all anesthetics so far available, including amyntal, have a depressive influence on glycogen formation in the liver. Furthermore, anesthesia and laparotomy, even if the greatest possible care is taken, are liable to be followed by an increased release of epinephrine from the adrenals.» This method has been adopted especially by French investigators and in work with larger laboratory animals, where for reasons of cost it is not possible to arrange extensive series.

Attempts to obtain continuous samples without placing the animals under narcosis have also been made. MOLITOR and POLLACK (1930) laid a lobe of the liver of rabbits and dogs outside the abdominal wall in accordance with a method indicated by KUNZ and MOLITOR (1929), after which they could take their samples. The method entails many sources of error and has not been used by others. A more promising experiment was made by CORI and CORI (1923), who constructed an abdominal window with a detachable lid, to be sewed into the abdominal wall of rabbits and dogs. A few days after this operation the experiment is made. The detachable part of the window is screwed off and samples can be repeatedly taken from the liver. The greatest drawback of this method is the tendency to hemorrhage. Moreover, the manipulations with the liver seem to lead to a continuous decrease in liver glycogen, also in control animals. The method was adopted and tested by several investigators, but was subjected to rather a severe criticism by several authors, including GREVENSTUK and LAQUEUR (1925 and 1926), EHRISMANN (1927) and HANDOWSKY (1928). CORI himself soon abandoned his method, and in 1931 he wrote: »A number of authors have used this method with moderate success. The difficulty was that stopping the hemorrhage by cautery, while very effective, proved to be an unsatisfactory procedure because the animal is severely damaged by absorption of toxic products. Recently it has been found that a high frequency current applied to the cut surface of the liver while it is being compressed stops the hemorrhage perfectly without causing more than a superficial

*Fig. 38. Survey of the most important and most often cited papers*

Author	Animal species	Time of fasting	Quantity of insulin	Time between injection and estimation
Dudley and Marrian (1923)	Mice	0	—	1 hr
Cori C. F. (1925)	•	17 hrs	0.01—0.4 U	15—60'
v. Meyenburg (1924)	•	24 hrs	—	—
Löw and Krema (1929)	•	0—16 hrs	0.5 U	1—1½ hr
Corkill (1930)	•	18—24 hrs	0.02—0.05 U	—
Tonutti and Wallruff (1938)	•	0	0.01 U	2 hrs
Barbour, Chaikoff, Macleod and Orr (1927)	Rats	24 hrs	1 U/kg	1 hr
	•	24 hrs	2 U/kg	1 hr
	•	24 hrs	3 U/kg	1 hr
Goldblatt (1929)	•	24 hrs	0.2—0.5 U	—
Löw and Krema (1929)	•	0—16 hrs	6 U	1—1½ hrs
Lawrence and McKane (1931)	•	24 hrs	0.1 U/100 gm	2 hrs
Daoud and Gohar (1933)	•	—	—	—
Russel (1938)	•	24 hrs	1—10 U per kg	4½ hrs
Spitzbarth (1940)	•	24 hrs	5 U	—
Goldblatt (1929)	•	0	—	—
Goldblatt (1933)	Guinea pig	24 hrs	1—2 U	—
Collazo, Händel and Rubino (1924)	•	18 hrs	7 U	4 hrs
Spitzbarth (1940)	•	24 hrs	4 U	—
Babkin (1923)	Rabbits	0	—	—
Dudley and Marrian (1923)	•	0	—	6 hrs

on the effect of insulin on the glycogen storage of the intact animal.

Effect on liver glycogen	Effect on muscle glycogen	Number of control animals	Number of insulin animals	
Decrease	—	3	3	Decapit. at first convulsion
Decrease	—	14	15	
No change	—	—	—	Histological examination. Practically no glycogen in the livers of control animals either
Decrease	Decrease or no change	—	—	Decapitated at beginning of convulsions
No change	Decrease	—	—	
No glycogen in the liver	—	—	—	Histological method
Decrease	Large decrease	—	—	Several animals. Different treatment
•	• •	—	—	
•	• •	—	—	
No change or slightly decreased	—	—	—	
Decrease	Decrease or no change	—	—	
Slight decrease	Slight decrease	9	8	
»Almost complete depletion»	»Considerable diminution»	—	—	»Convulsive doses»
Decrease	Increase	10	34	The animals are given glucose simultaneously with insulin injection
Large decrease	•	3	3	
Increase	—	—	—	Food <i>ad lib.</i> until decap
No change	—	—	—	Certain animals
Increase	Increase	—	—	Glucose simultaneously with insulin injection
Decrease	•	3	3	
»Markedly lowered»	»Markedly lowered»	2	2	
Decrease	Large decrease	3	3	Decapitation at first convulsion

Author	Animal species	Time of fasting	Quantity of insulin	Time between injection and estimation
Brugsch, Benatt, Horsters and Katz (1924)	Rabbits	24 hrs	—	—
Heymans and Heymans (1925)	»	—	—	—
Bonn (1925)	»	24 hrs	50 U	2-3 hrs
Frank, Nothmann and Hartmann (1925 and 1927)	»	3-6 d.	0.1 U/kg	3-4 hrs
v. Meyenburg (1924)	»	24 hrs	—	4-5 hrs
Grevenskuk and Laquer (1926)	»	4 d.	0.1 U/kg	2 hrs
Visco (1926)	»	5 d.	—	—
Markowitz (1926)	»	5-10 d.	—	—
Ehrismann (1927)	»	1-4 d.	—	—
Handowsky (1928)	»	0-24 hrs	Varying	—
Rossi (1928)	»	5-10 d.	5 U.	—
Sahyun and Luck (1929)	»	24 hrs	25 U/kg	1 hr
Goldblatt (1929)	»	24 hrs	0.5 U	1-3 hrs
	»	24 hrs	0.2 U	1-3 hrs
	»	24 hrs	0.5 U	1-3 hrs
Goldblatt (1930)	»	48 hrs	0.2-1 U	2-5 hrs
Corkill (1930)	»	24 hrs	0.5 U	—
Loeb, Nichols and Paige (1931)	»	0-2 d.	7-75 U/kg i. v.	—
Corkill, Marks and White (1933)	»	24 hrs	—	—

Effect on liver glycogen	Effect on muscle glycogen	Number of control animals	Number of insulin animals	
Large decrease in convulsive state	—	—	1	
Decrease	—	—	—	Convulsions thought to give decrease in liver glycogen
No change	No change	2	3+3	» Convulsive doses »
Considerable increase	No change	7	11	Blood sugar lowered. No convulsions
Considerable increase	—	—	—	Histological examination
Sometimes increase, sometimes decrease	—	—	—	
Large increase	—	—	—	Abdominal window
” ”	—	—	—	Several small insulin injections during fasting
No increase	—	—	—	Several animals. Different treatment
—	Decrease	68	16	Abdominal window d = 2 ε
Disappeared	Disappeared	—	—	»... getötet sobald die Insulin-wirkung ihre Höhepunkt erreicht hatte.
No change	No change	4	3	
Considerable increase	No certain changes	3	3	10 weeks old
Considerable increase	No certain changes	3	3	6 > >
Considerable increase	Decrease	3	3	6 > > . Severe convulsions
Increase	No change	14	16	
Considerable increase	Increase	11	11	Decapitated as soon as flaccid and coordination lost
Decrease	—	—	—	
Increase	—	—	—	

Author	Animal species	Time of fasting	Quantity of insulin	Time between injection and estimation
Goldblatt (1933)	Rabbits	24 hrs	1 U/700 gm	—
Sunaba (1936)	»	24 hrs	0.5—2 U/kg	—
Bridge (1938)	»	12 hrs	1—4 U/kg	6 hrs
Brentano (1939)	»	24 hrs	5 U/kg	5 hrs
Goldblatt (1930)	Cat	2—5 d.	0.2—1 U	2—5 hrs
Reid (1936)	»	48 hrs	—	2—3 hrs
Fisher and Lackey (1925)	Dog	0	—	—
Ehrismann (1927)	»	24 hrs	—	2 hrs
Bürger and Kohl (1935)	»	—	0.5 U/kg	—12 hrs
Vendég (1935)	»		0.05—0.1 U 0.1—0.5 U 0.5—1 U/kg	— — —
Hebb (1937)	»	12—48 hrs	—	—
Corkill (1930)	Ferrets	24 hrs	12 U	3 hrs
Rathery, Gibert and Laurent (1930)	Monkey	12 hrs	repeated small doses	17—25 hrs
Rathery, Gibert and Laurent (1931)	»	—	0.75 U	—
Christol, Hédon, Loubatières and Monnier (1938)	»	0	repeated small doses	—
		0	3 U/kg	—

Effect on liver glycogen	Effect on muscle glycogen	Number of control animals	Number of insulin animals	
Increase	Slight decrease	—	—	
Decrease	Decrease	—	—	Sampling in narcosis
“	Increase	8	16	Intravenous infusion of insulin and glucose
“	“	—	—	Glucose simultaneously
Increase	No change	—	—	
No certain change	—	—	—	Infusion of insulin intravenously, 0.07—0.28 U/kg and hr
Decrease	—	3	1	
No increase	—	—	—	Abdominal window
Decrease	—	14	—	Sampling in pernocton anaesthesia
Slight increase	—	—	—	
Decrease	—	14	—	Sampling under pernocton anaesthesia. Insulin and glucose injected intraven
Considerable decrease	—	—	—	
Decrease	—	3	3	Given insulin and dextrose. Sampling under anaesthesia
No change	Increase	3	3	
Decrease	Decrease	4	—	Sampling under anaesthesia
“	No change	—	—	Sampling under anaesthesia
Slight increase	—	3	—	Glucose simultaneously
Decrease	—	—	—	Sampling under anaesthesia
			—	Sampling under anaesthesia

ncrosis. Even with this improvement scrial dcterminations on rats seem preferable to taking samples of liver from one and the same animal.»

### B. Experiments with series of insulin-treated animals and control animals

have proved to be the working method which has yielded the best results in studying the effect of insulin on the glycogen depots. Also here, however, there are many lurking sources of error (see the immediately preceding section of this work). In particular, investigators do not seem to have realized the magnitude of the biological variation and have accordingly worked with too small series. The biological variation, however, is of fundamental importance. LAQUEUR and DE JONGH (1925) clearly explain the immense importance of this factor. They state: »...ergibt sich aus dem obigen Material die Sinnlosigkeit, aus *ein* oder *drei* Versuchen Schlüsse zu ziehen . . . Denn, wenn sich herausstellt, dass die 'Streuungsbreite' eine schr bedeutende ist, dann dürfen einerseits Untersuchungen an einem oder drei Tieren nich gemacht werden, andererseits, wenn dies noch geschieht, brauchen sie jedenfalls nicht mehr gelesen werden.» These points of view, as may be gathered from the following summary, have unfortunately been entirely disregarded by a large number of investigators.

In works on this subject statistical treatment of the material is often non-existing, so that the authors have no clear idea as to what conclusions their experiments entitle them to draw. In some extremely rare cases the conclusions drawn are not sufficiently far-reaching, but in most publications the reverse is the fact.

In Fig. 38 the principal and most frequently cited of the papers on the effect of insulin on the glycogen stores, made by direct analysis of organs in experiments on intact normal animals, are tabulated.

Very thorough investigations into the effect of insulin on the body glycogen have been made by CORI and CORI and their co-workers. With the aid of the previously mentioned abdominal

window, CORI, CORI, and PUCHER (1923) studied the effects of insulin on the liver glycogen of a rabbit and arrived at the result that »Iletin causes glycogen synthesis during ingestion of glucose even though the blood sugar and free liver sugar is below that of a normal starved animal». These experiments, however, were few in number and not quite convincing. C. F. CORI (1925) states, as a result of studies on rabbits with the abdominal window method: »Our data on the influence of insulin on the liver glycogen of starving rabbits show that there is no appreciable change in the glycogen or total carbohydrate content within the first hour of insulin action, whether the initial glycogen content is high or low, or whether the fall in blood sugar is slight or strong. In the second to sixth hour of insulin action the glycogen content of the liver may remain constant or may decrease.» He also examined mice which had received 0.01—0.04 U. of insulin. It was found that they reacted much quicker, for which reason all the analyses were made 15—60 minutes after the insulin injection. »The average glycogen content of the liver of the injected mice was 39 per cent lower than the average of the corresponding controls. In only one half of these experiments did a decrease in the glycogen content of the liver of the injected mice occur, while the other half showed no change in the liver glycogen after the injection of insulin.» In regard to the animals which had concurrently received glucose, he writes: »In summarizing this section the most important fact seems to be that the insulin-injected animal not only synthesizes glycogen in the liver, when it has an excess of sugar available, but that the rate of glycogen deposition is actually increased». Normal fasting animals who receive insulin, on the other hand, show no increase in the liver glycogen content, which is interpreted to be due to the fact that they have no sugar available for storage in the form of glycogen. »It is concluded that insulin produces glycogen synthesis whenever there is a certain excess of sugar available.»

The abdominal window method was later abandoned after severe criticism by several authors.

CORI (1926) and CORI and CORI (1926 and 1928) worked with rats which had fasted for 24—48 hours. They received glucose through a tube in an amount which guaranteed resorption in at least

four hours. These investigators computed the combustion, the amount of glucose resorbed and the amount of glycogen deposited. »The average amount of sugar absorbed was nearly the same in both series.» The animals which had received insulin deposit less glycogen in the liver and more in the rest of the body than the controls. CORI and CORI (1928) supplement their above reported experiments on the effect of insulin in the »absorptive state», with investigations in the »postabsorptive state». After 24 hours fasting the rats receive through a tube, per 100 g. of weight, 2.5 cc of a glucose solution containing 1.065 mg glucose. This is the amount of glucose which on an average is resorbed in four hours under the said conditions. Four hours after feeding, the insulin injection experiments begin. The amount of glycogen which the rats at this time should show is estimated in accordance with previous experiments. The changes in the course of three hours are determined. »The insulinized rats utilized three times more liver glycogen but only slightly more body glycogen than the control rats... According to this analysis, insulin is a hormone that leads to a preferential utilization of blood sugar and indirectly of liver glycogen, the latter being the only important source of blood sugar in the body.» CORI and CORI (1929) determined the effect of insulin on rats concurrently with intravenous infusion of glucose in large amounts. »For an equal quantity of glucose injected, the insulinized animals showed a blood sugar level and a glycogen content of the liver approximately one half of that of the control animals. When a similar blood sugar level was maintained in control and insulinized animals by injecting less glucose into the former than into the latter there was no difference in the amount of glycogen deposited in the two groups of animals.» CORI and CORI (1929), after experiments on rats which had received insulin after fasting, conclude: »Die Insulinwirkung ist am Hungertier nicht sehr Eindrucksvoll, was bei dem geringen Glykogenbestand der Leber nicht zu verwundern ist. Denn Insulin erhöht die Kohlehydratverbrennung vor allem auf Kosten des Leberglykogens, und zwar in der Weise, dass infolge der gesteigerten Aufnahme von Blutzucker in den peripheren Geweben mehr Zucker von der Leber nachgeliefert wird. Muskelglykogen wird erst dann in verstärktem Masse angegriffen, wenn der Glykogen-

bestand der Leber niedrig ist.» CORI, CORI and BUCHWALD (1930), in regard to non-fasting rats which had received 0.24 units per 100 g, state: »15 minutes after the insulin injection liver glycogen and blood sugar were unchanged, while after one hour both had diminished.»

### Summary.

As appears from the above account, there is a considerable divergence of opinion regarding the effect of insulin on the glycogen storage. Most of the investigations were made on very few animals and are therefore, in view of the marked individual variations, not very convincing, even if they sometimes perhaps contain correct observations. But also the investigations which have been made on a larger number of animals have given contradictory results. Thus, for example, it is stated in regard to the liver that insulin entails an increase in the glycogen storage (FRANK, NOTHMANN and HARTMANN; GOLDBLATT; CORKILL; VENDÉG;) whereas a decrease is reported by CORI and CORI, LAWRENCE and McCANE, RUSSEL, BRIDGE, BÜRGER and KOHL, VENDÉG. As regards the muscles an increase was noted by RUSSEL, CORKILL, BRIDGE and CORI and CORI, no change by FRANK, NOTHMANN and HARTMANN, GOLDBLATT, whilst e.g. HANDOWSKY as well as LAWRENCE and McCANE found that insulin tended to lower the muscle glycogen content.

It is difficult to say to what these contradictory results may have been due, but they may partly be explained by the fact that the experimental conditions were not comparable. As a rule merely the effect of a single dosage was examined and the result of it was analyzed some time after the injection. Merely sporadic attempts to study different dosages under the same conditions were made by VENDÉG, GOLDBLATT, CORI and CORI. These experiments, however, never comprised sufficiently varying dosages and they were never extended to analyses at different times after the injection, except in experiments on narcotized animals where samples of the liver were repeatedly taken during the test.

It is impossible by studies of the literature to arrive at a reliable view regarding the effect of insulin in excess on the glycogen storage of normal animals.

## Effect of insulin on the glycogen stores in mice

Most of the experiments were made on mice, as these animals had shown the greatest uniformity in their reaction. This had also the great advantage that the whole animal can be used for analysis.

The experiments were divided into four groups: 1. The mice received the insulin injection without preceding fast. After the injection they were starved until decapitation. 2. The mice received the insulin injection after a fast of 24 hours and were afterwards starved until decapitation. 3. The mice received the injection after 24 hours fasting and then had free access to food. 4. In some of the mice such small doses of insulin were injected that the blood sugar was not affected. The planning of the experiments is indicated by the table in Fig. 39.

The experiments in group 1. were made in order to study the effect of insulin on as »normal» an organism as possible; those in group 2. in order to study the effect on an organism with reduced glycogen content and without a supply of carbohydrates in the intestinal canal. In the literature it has in fact been contended that the effect of insulin on the glycogen depots is to a certain extent dependent on the supply of carbohydrates. The experiments in group 4 were made in order to control the statements of FRANK, NOTHEMANN and HARTMANN (1925 and 1927) and others that insulin in very small doses entails a storage of glycogen in the liver.

The condition of the mice at the time of decapitation can be roughly described as follows: 1. Unaffected. 2. Slightly

*Fig. 39. Table showing the planning of the experiments on normal mice. The table gives the treatment, the insulin doses and the time of insulin injection. All animals decapitated at 8 a.m.*

Treatment	Time of injection	7.35 a.m.	7 a.m.	6 a.m.	4 a.m.	12 p.m.
Food <i>ad lib.</i> After insulin injection no food.		0.004; 0.008; 0.02; 0.2;	0.004; 0.008; 0.02; 0.2;	— 0.02;	0.008; 0.2;	0.004; 0.008; 0.02; 0.2;
24 hrs fasting. After insulin injection no food.				0.004; 0.008; 0.02; 0.2;	0.004; 0.008; 0.02; 0.2;	0.004; 0.008; 0.02; 0.2;
24 hrs fasting. After insulin injection food <i>ad lib.</i>				0.004; 0.02; 1	0.004; 0.008; 0.02; 1	0.004; 0.008; 0.02; 0.2; 1
Food <i>ad lib.</i> After insulin injection no food.				0.001; 0.002;		

a f f e c t e d : apathetic, sit still and cowering in a corner, but not paralyzed. 3. G r e a t l y a f f e c t e d : more or less paralyzed, unconscious, considerable fall of temperature, spasms.

**Group 1.** Those doses were chosen because a dosage of 0.004 U. merely caused a slight reduction of the blood sugar without affecting the general condition, whereas the dosage 0.2 U. affected it in a very marked degree. This latter dosage, however, was not so large that it led to death, at any rate not within the time covered by the experiments.

None of the mice died from these insulin doses. With a dosage of 0.004 U. insulin subcutaneously all the animals remain throughout completely unaffected. With a dosage of 0.008 U. some of the mice were slightly affected after two hours. After the lapse of four hours or longer all of them seemed to be unaffected. The dosage 0.02 U. slightly affects some of the mice after one hour, markedly affects a few of them after two hours and slightly affects the majority of them. A few of the mice still remain unaffected. After four hours all the mice appear to be lively and unaffected. The dosage 0.2 U. after one hour already affects all the mice, most of them slightly, a few markedly. After two and four hours they are more strongly affected. Eight hours after the injection some of the mice are still slightly affected, whilst the others are unaffected. Such a short time as 25 minutes after the injection all the mice are unaffected by all the dosages.

The general condition of these animals is thus most affected 2—4 hours after the insulin injection. The effect sets in earlier and continues longer, the larger the dose is.

*The variations in the liver glycogen* are shown in the tables Figs. 40—49 and in the diagram Fig. 50. The diagram shows clearly that all the insulin dosages employed, give reduction of the liver glycogen content. 25 minutes after the injection the tendency to reduction is noticeable as regards all the dosages and after 1—2 hours it reaches a maximum. Afterwards the liver glycogen content again begins to rise. The tables show that the reduction of the liver glycogen content 1 hour after the injection is so marked that the difference between the controls and the insulinized animals is statistically significant for all the

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故其子曰：「吾父之子，其名何也？」

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$\mu_1$	$\mu_2$	$\mu_3$
0.0	0.0	0.0
0.2	0.0	0.0
0.4	0.0	0.0
0.6	0.0	0.0
0.8	0.0	0.0
1.0	0.0	0.0
1.2	0.0	0.0
1.4	0.0	0.0
1.6	0.0	0.0
1.8	0.0	0.0
2.0	0.0	0.0
2.2	0.0	0.0
2.4	0.0	0.0
2.6	0.0	0.0
2.8	0.0	0.0
3.0	0.0	0.0
3.2	0.0	0.0
3.4	0.0	0.0
3.6	0.0	0.0
3.8	0.0	0.0
4.0	0.0	0.0
4.2	0.0	0.0
4.4	0.0	0.0
4.6	0.0	0.0
4.8	0.0	0.0
5.0	0.0	0.0
5.2	0.0	0.0
5.4	0.0	0.0
5.6	0.0	0.0
5.8	0.0	0.0
6.0	0.0	0.0
6.2	0.0	0.0
6.4	0.0	0.0
6.6	0.0	0.0
6.8	0.0	0.0
7.0	0.0	0.0
7.2	0.0	0.0
7.4	0.0	0.0
7.6	0.0	0.0
7.8	0.0	0.0
8.0	0.0	0.0
8.2	0.0	0.0
8.4	0.0	0.0
8.6	0.0	0.0
8.8	0.0	0.0
9.0	0.0	0.0
9.2	0.0	0.0
9.4	0.0	0.0
9.6	0.0	0.0
9.8	0.0	0.0
10.0	0.0	0.0

卷之三

*Fig. 42. Table. Normal mice. Females. Food ad lib. No food after insulin injection at 7 a. m. Decapitation at 8 a. m.*

Dose	No. of animals	Body weight gm		Liver weight mgm		Alim. canal and spleen mgm		Liver glycogen per cent		Body glycogen per cent		$\bar{X} \pm \epsilon_{\bar{X}}$	$\bar{X} \pm \epsilon_{\bar{X}}$
		No. of animals	Body weight gm	$\bar{X} \pm \epsilon_{\bar{X}}$	Liver weight mgm	$\bar{X} \pm \epsilon_{\bar{X}}$	Alim. canal and spleen mgm	$\bar{X} \pm \epsilon_{\bar{X}}$	Liver glycogen per cent	$\bar{X} \pm \epsilon_{\bar{X}}$	Body glycogen per cent	$\bar{X} \pm \epsilon_{\bar{X}}$	
Controls	12	19.8 ± 0.6	943 ± 34	2 553 ± 133	2.88 ± 0.18	0.085 ± 0.008	(n = 11)	24.1 ± 1.7	(n = 11)	14.6 ± 2.0			
0.004	12	18.8 ± 0.6	866 ± 46	2 222 ± 109	1.46 ± 0.32	0.071 ± 0.004				11.2 ± 1.4			
0.008	12	20.1 ± 0.6	918 ± 77	2 532 ± 135	0.93 ± 0.20	0.066 ± 0.006				15.5 ± 2.2			
0.02	12	18.8 ± 0.8	876 ± 37	2 405 ± 81	1.72 ± 0.31	0.063 ± 0.007				9.3 ± 1.0			
0.2	12	18.6 ± 0.7	863 ± 33	2 398 ± 96	0.57 ± 0.12	0.069 ± 0.009							

*Fig. 43. Table showing the difference between controls and insulinized animals in Fig. 42.*

Dose	Liver glycogen				Body glycogen				Glycogen content in gm per 10 gm of body weight minus alim. canal and spleen			
	$d \pm \epsilon_d$	t	df	P	$d \pm \epsilon_d$	t	df	P	$d \pm \epsilon_d$	t	df	P
0.004	1.42 ± 0.37	3.838	22	< 0.001	0.014 ± 0.009	1.555	21	0.2 — 0.1	9.5 ± 2.6	3.654	21	0.01 — 0.001
0.008	1.95 ± 0.27	7.222	22	< 0.001	0.019 ± 0.009	2.111	22	0.05 — 0.02	12.9 ± 2.2	5.864	22	< 0.001
0.02	1.16 ± 0.36	3.222	22	0.01 — 0.001	0.022 ± 0.011	2.000	22	0.1 — 0.05	8.6 ± 2.8	3.072	22	0.01 — 0.001
0.2	2.31 ± 0.22	10.500	22	< 0.001	0.016 ± 0.012	1.338	22	0.2	14.8 ± 2.0	7.400	22	< 0.001

Fig. 14. Table. Normal mice, female, d<sub>0</sub> and d<sub>16</sub>. No food after i.v. injection of 0.4% propylthiouracil at 0, 0.1, 0.2, 0.5.

Dose	No. of animals	Body weight (g)	Liver weight (mg)	After oral administration		After injection of 0.4% propylthiouracil per cent. increase in liver weight		Glycogen content in mg/g liver per cent. increase in liver weight	
				$\bar{x} \pm S_{\bar{x}}$	$S_{\bar{x}} \pm S_{\bar{S}}$	$\bar{x} \pm S_{\bar{x}}$	$S_{\bar{x}} \pm S_{\bar{S}}$	$\bar{x} \pm S_{\bar{x}}$	$S_{\bar{x}} \pm S_{\bar{S}}$
Control	17	21.4 ± 0.1	0.21 ± 0.01	21.0 ± 0.2	0.20 ± 0.01	21.6 ± 0.2	0.22 ± 0.01	21.9 ± 0.1	0.24 ± 0.01
0.05	17	20.3 ± 0.2	0.24 ± 0.02	20.6 ± 0.2	0.25 ± 0.02	20.5 ± 0.2	0.26 ± 0.02	20.5 ± 0.2	0.26 ± 0.02
0.02	17	21.1 ± 0.3	0.21 ± 0.03	20.9 ± 0.3	0.20 ± 0.03	21.2 ± 0.2	0.23 ± 0.02	21.4 ± 0.2	0.24 ± 0.02
0.5	16	21.7 ± 0.2	0.27 ± 0.02	21.5 ± 0.2	0.26 ± 0.02	21.5 ± 0.2	0.25 ± 0.02	21.5 ± 0.2	0.25 ± 0.02

Fig. 15. Table showing the difference between control and treated animals obtained from the data in Fig. 14.

Dose	Liver effects				Fatty changes				Glycogen content in mg/g liver per cent. increase in liver weight			
	$\bar{x} \pm S_{\bar{x}}$	$t$	$P$	$d = t \cdot S_{\bar{x}}$	$t$	$P$	$d = t \cdot S_{\bar{x}}$	$t$	$P$	$d = t \cdot S_{\bar{x}}$	$t$	$P$
0.004	2.02 ± 0.24	7.314	< 0.001	0.037 ± 0.039	1.433 ± 0.32	0.1 ± 0.05	0.15 ± 0.05	11.5 ± 2.21	0.001	0.03 ± 0.03	0.1 ± 0.03	0.001
0.02	2.31 ± 0.27	9.657	< 0.001	0.015 ± 0.018	2.673 ± 0.72	0.1 ± 0.05	0.19 ± 0.05	6.619 ± 1.54	0.001	0.03 ± 0.03	0.1 ± 0.03	0.001
0.2	2.37 ± 0.25	9.015	< 0.001	0.026 ± 0.021	2.000 ± 0.31	0.1 ± 0.05	0.21 ± 0.05	6.551 ± 0.71	0.001	0.03 ± 0.03	0.1 ± 0.03	0.001

*Fig. 46. Table. Normal mice. Females. Food ad lib. No food after insulin injection at 4 a. m. Decapitation at 8 a. m.*

Dose	No. of animals	Body weight gm $\bar{X} \pm \epsilon_{\bar{X}}$	Liver weight gm $\bar{X} \pm \epsilon_{\bar{X}}$	Alim. canal and spleen gm $\bar{X} \pm \epsilon_{\bar{X}}$	Liver glycogen per cent $\bar{X} \pm \epsilon_{\bar{X}}$		Body glycogen per cent $\bar{X} \pm \epsilon_{\bar{X}}$	Glycogen content in mgm per 10 gm of body weight minus alim. canal and spleen $\bar{X} \pm \epsilon_{\bar{X}}$
					d	$\pm \epsilon_d$		
Controls	12	20.9 ± 0.6	954 ± 28	2 420 ± 114	2.95	± 0.43	0.083	± 0.008
0.004	12	22.2 ± 0.8	993 ± 38	2 294 ± 70	2.60	± 0.26	0.069	± 0.006
0.008	12	21.8 ± 0.5	958 ± 43	2 359 ± 53	1.78	± 0.23	0.078	± 0.008
0.02	12	21.2 ± 0.5	879 ± 28	2 210 ± 98	0.61	± 0.10	0.062	± 0.007
0.2	12	21.7 ± 0.5	917 ± 28	2 491 ± 105	0.34	± 0.03	0.088	± 0.008

*Fig. 47. Table showing the difference between controls and insulinized animals tabulated in Fig. 46.*

Dose	Liver glycogen						Body glycogen				Glycogen content in mgm per 10 gm of body weight minus alim. canal and spleen				
	d	$\pm \epsilon_d$	t	df	P	d	$\pm \epsilon_d$	t	df	P	d	$\pm \epsilon_d$	t	df	P
0.004	0.35	± 0.50	0.700	22	0.5 — 0.4	0.015	± 0.010	1.600	22	0.2 — 0.1	3.4	± 2.8	1.214	22	0.3 — 0.2
0.008	1.17	± 0.49	2.388	22	0.05 — 0.02	0.006	± 0.011	0.456	22	0.7 — 0.6	6.8	± 2.9	2.345	22	0.05 — 0.02
0.02	2.34	± 0.45	5.200	22	< 0.001	0.021	± 0.011	1.909	22	0.1 — 0.05	14.5	± 2.5	5.800	22	< 0.001
0.2	2.61	± 0.43	6.070	22	< 0.001	- 0.006	± 0.011	0.456	22	0.7 — 0.6	13.3	± 2.6	5.115	22	< 0.001

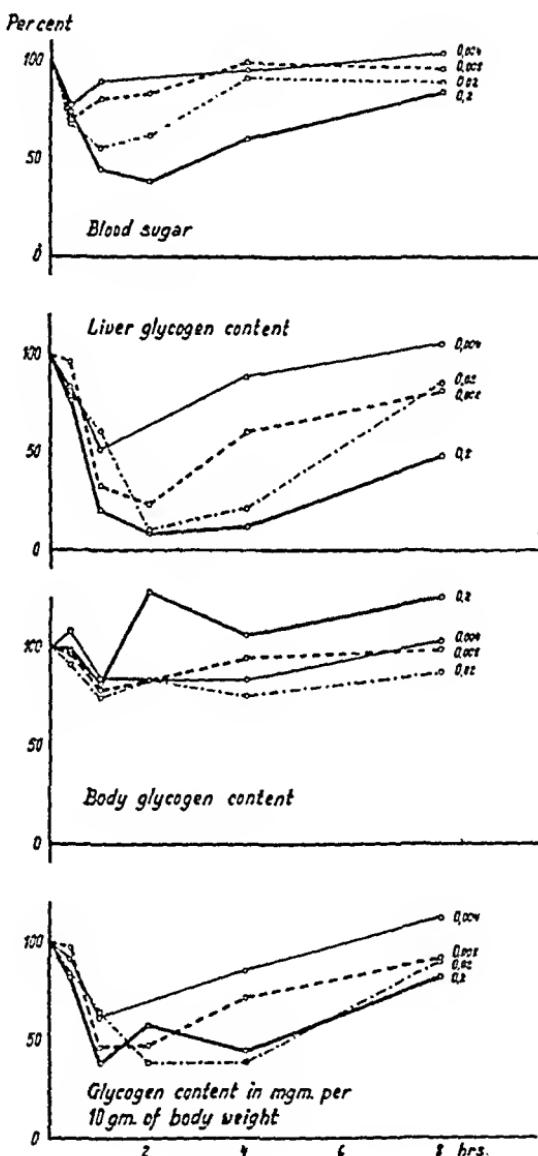
Fig. 48. Table. Normal mice. Females. Food ad lib. No food after insulin injection at 12 p. m. Decapitation at 8 a. m.

Dose	No. of animals	Body weight gm $\bar{X} \pm \epsilon_{\bar{X}}$	Liver weight mgm $\bar{X} \pm \epsilon_{\bar{X}}$	Alim. canal and spleen mgm $\bar{X} \pm \epsilon_{\bar{X}}$	Liver glycogen per cent $\bar{X} \pm \epsilon_{\bar{X}}$		Body glycogen per cent $\bar{X} \pm \epsilon_{\bar{X}}$	Glycogen content in mgm per 10 gm of body weight minus alim. $\bar{X} \pm \epsilon_{\bar{X}}$	
Controls	10	19.5 ± 0.5	955 ± 57	2,673 ± 115	2.21 ± 0.33	0.077 ± 0.001	19.8 ± 2.3		
0.004	10	18.7 ± 0.6	965 ± 79	2,440 ± 142	2.32 ± 0.11	0.079 ± 0.006	22.2 ± 3.4		
0.008	10	19.1 ± 0.4	913 ± 51	2,484 ± 123	1.79 ± 0.42	0.077 ± 0.009	18.3 ± 3.7		
0.02	10	18.4 ± 0.4	891 ± 46	2,426 ± 142	1.88 ± 0.13	0.067 ± 0.006	17.8 ± 3.4		
0.2	10	17.9 ± 0.5	916 ± 60	2,469 ± 136	1.06 ± 0.29	0.037 ± 0.008	16.2 ± 2.1		

Fig. 49. Table showing the difference between controls and insulinized animals tabulated in Fig. 48.

Dose	Liver glycogen						Body glycogen				Glycogen content in mgm per 10 gm of body weight minus alim. canal and spleen			
	$d \pm \epsilon_d$	t	df	P	$d \pm \epsilon_d$	t	df	P	$d \pm \epsilon_d$	t	df	P		
0.004	-0.11 ± 0.52	0.212	18	0.9 — 0.8	-0.002 ± 0.007	0.286	18	0.8 — 0.7	-2.4 ± 4.1	0.585	18	0.6 — 0.5		
0.008	0.42 ± 0.53	0.792	18	0.5 — 0.4	0	—	—	—	1.5 ± 4.3	0.349	18	0.8 — 0.7		
0.02	0.33 ± 0.54	0.611	18	0.6 — 0.5	0.010 ± 0.007	1.429	18	0.2 — 0.1	2.0 ± 4.1	0.488	18	0.7 — 0.6		
0.2	1.15 ± 0.44	2.614	18	0.02 — 0.01	-0.020 ± 0.009	2.222	18	0.05 — 0.02	3.6 ± 3.4	1.059	18	0.3		

*Fig. 50. Diagrams showing the effect of different insulin doses on blood sugar and storage of glycogen at different lengths of time after injection. Food ad lib. After insulin injection no food. All animals decapitated at 8 a. m. All values given in per cent of the corresponding values of the controls.*



dosages; likewise 2 hours after the injection. 4 hours after the injection only the two largest dosages show a statistically significant difference as compared with the controls, whilst the difference for the dosage 0.008 U. must be designated as probable ( $P = 0.05 - 0.02$ ). In the animals which had received the smallest dosage, 0.004 U., the liver glycogen content by that time had risen so much that it showed a good correspondence with that of the controls. 8 hours after the injection all the animals except those which had received the largest dosage showed good correspondence with the controls. As regards the animals which had received a dosage of 0.2 U., a reduction is still statistically probable ( $P = 0.02 - 0.01$ ).

*Variations in the body glycogen content:* The diagram shows a slight reduction of the body glycogen content for all dosages 1 hour after the injection. Afterwards we note as regards the dosage 0.2 U. a marked rise, so that these animals 2 hours after the injection show a body glycogen content exceeding that of the controls. The variations after the other dosages are less pronounced. It is evident from the tables that the insulinized animals 25 minutes after the injection showed a good correspondence with the controls. 1 hour after the injection there is a difference bordering on probability ( $P = 0.05 - 0.01$ ) between the controls and the animals which had received 0.008 U. 2 hours after the injection there is no convincing difference between any of the insulin series and the controls. If the body glycogen content in the animals which had received 0.2 U. and in those which had received 0.02 U. is compared, we find a difference of  $0.040\% \pm 0.014$  ( $t = 2.857$ ;  $df = 31$ ;  $P = 0.01 - 0.001$ ), which must be regarded as statistically very probable, if not certain. 4 hours after the injection there is no statistical difference for any of the dosages as compared with the control animals. 8 hours after the injection the animals which had received the largest dosage showed a statistically probable increase as compared with the controls ( $P = 0.05 - 0.02$ ). The animals which had received the other insulin dosages show good correspondence with the controls.

*Variations in the total amount of glycogen:* It will be seen from the diagram that all the dosages employed result in a reduction of the body glycogen stores. The diminution culminates after

1—4 hours and then slows down, so that all the animals 8 hours after the injection show a good correspondence with the controls. One hour after the injection the diminution is statistically significant as regards all the dosages; likewise after 2 hours.

As regards the *blood sugar* (cf the diagram) one can observe as regards all the dosages a falling tendency 25 minutes after the injection. After the lapse of one hour the blood sugar level has again begun to rise after the two smallest dosages, whereas it still falls after the two larger ones. Four hours after the injection the blood sugar level in the insulinized animals is again about normal except in those which had received the largest dosage, 0.2 U., in which it is still lowered. Eight hours after the injection even these animals begin to show a normal blood sugar content.

*Recapitulation:* The effect of insulin on the general condition of the animals and on the blood sugar level is proportional to the dosage. It sets in more rapidly and continues longer, the larger the dosage.

All the insulin dosages tend to lower the liver glycogen content. The largest doses give an increase of the body glycogen content. The total amount of glycogen per weight unit of the animal diminishes under the effect of insulin.

**Group 2.** The results of these investigations have been tabulated in Figs. 51—58 and are graphically shown in the diagram Fig. 59.

*Effect on the general condition:* One hour after the injection all the mice seemed to be still unaffected. Two hours after the injection most of those which had received the dosage 0.02 U. are slightly affected, whilst those which had received 0.2 U. are greatly affected. After 4 and 8 hours respectively a few of the mice are slightly affected by these larger dosages. After the dosages 0.004 and 0.008 U. they appear to be unaffected.

*The variations in the liver glycogen*, as shown by the diagram, are essentially similar to that of the animal in the preceding experimental group. It will be seen from the tables that the reduction one hour after the injection must be regarded as statistically probable for the dosage 0.004 U. ( $P = 0.05 - 0.02$ ) and as statistically significant for the other dosages. The situation is the same

Fig. 51. Table. Normal mice. Females. 24 hrs. fasting. No food after insulin injection at 7 a. m. Decapitation at 8 a. m.

Dose	No. of animals	Body weight gm $\bar{X} \pm \varepsilon_{\bar{X}}$	Liver weight mgm $\bar{X} \pm \varepsilon_{\bar{X}}$	Alim. canal and spleen mgm $\bar{X} \pm \varepsilon_{\bar{X}}$	Liver glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$	Body glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$	Glycogen content in gm per 10 gm of body weight minus alim. canal and spleen $\bar{X} \pm \varepsilon_{\bar{X}}$
Controls	12	18.1 ± 1.1	867 ± 65	2 272 ± 120	0.57 ± 0.06	0.037 ± 0.006 (n = 11)	6.9 ± 1.1
0.004	10	17.7 ± 1.0	866 ± 58	2 296 ± 92	0.40 ± 0.05	0.033 ± 0.006	5.6 ± 0.8
0.008	12	18.1 ± 0.8	755 ± 52	2 183 ± 100	0.36 ± 0.03	0.036 ± 0.004	5.1 ± 0.4
0.02	12	17.7 ± 0.6	891 ± 87	2 308 ± 78	0.30 ± 0.03	0.039 ± 0.003	5.4 ± 0.5
0.2	12	17.8 ± 0.7	793 ± 55	2 218 ± 89	0.26 ± 0.02	0.047 ± 0.004	5.7 ± 0.5

Fig. 52. Table showing the difference between controls and insulinized animals tabulated in Fig. 51.

Dose	Liver glycogen						Body glycogen				Glycogen content in gm per 10 gm of body weight minus alim. canal and spleen			
	d ± ε_d	t	df	P	d ± ε_d	t	df	P	d ± ε_d	t	df	P		
0.004	0.17 ± 0.08	2.125	20	0.05 — 0.02	0.004 ± 0.008	0.500	19	0.7 — 0.6	1.4 ± 1.4	1.000	19	0.4 — 0.3		
0.008	0.21 ± 0.07	3.000	22	0.01 — 0.001	0.001 ± 0.007	0.143	21	0.9 — 0.8	1.8 ± 1.2	1.560	21	0.2 — 0.1		
0.02	0.27 ± 0.07	3.857	22	< 0.001	-0.002 ± 0.008	0.260	21	0.9 — 0.8	1.6 ± 1.2	1.260	21	0.3 — 0.2		
0.2	0.32 ± 0.06	5.383	22	< 0.001	-0.010 ± 0.007	1.429	21	0.2 — 0.1	1.2 ± 1.2	1.000	21	0.4 — 0.3		

Fig. 53. Table. Normal mice. Females. 24 hrs. fasting. No food after insulin injection at 6 a. m. Decapitation at 8 a. m.

Dose	No. of animals	Body weight gm $\bar{X} \pm \varepsilon_{\bar{X}}$	Liver weight mgm $\bar{X} \pm \varepsilon_{\bar{X}}$	Alim. canal and spleen mgm $\bar{X} \pm \varepsilon_{\bar{X}}$		Liver glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$	Body glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$	Glycogen content in mgm per 10 gm of body weight minus alim. canal and spleen $\bar{X} \pm \varepsilon_{\bar{X}}$
Controls	15	18.3 ± 0.2	715 ± 16	1765 ± 45	0.44 ± 0.08 (n = 14)	0.039 ± 0.003		5.8 ± 0.6 (n = 14)
0.004	17	18.7 ± 0.2	719 ± 18	1820 ± 26	0.26 ± 0.05 (n = 15)	0.037 ± 0.002		4.6 ± 0.4 (n = 15)
0.008	17	20.4 ± 0.5	856 ± 46	2210 ± 89	0.19 ± 0.01	0.034 ± 0.001		4.2 ± 0.3
0.02	15	19.0 ± 0.3	750 ± 17	1902 ± 66	0.17 ± 0.008	0.044 ± 0.008		5.0 ± 0.3
0.2	16	18.8 ± 0.2	771 ± 17	1843 ± 22	0.16 ± 0.03	0.051 ± 0.003		5.6 ± 0.3

Fig. 54. Table showing the difference between controls and insulinized animals tabulated in Fig. 53.

Dose	Liver glycogen						Body glycogen			Glycogen content in mgm per 10 gm of body weight minus alim. canal and spleen
	$d \pm \varepsilon_d$	t	df	P	$d \pm \varepsilon_d$	t	df	P		
0.004	0.18 ± 0.09	2.000	27	0.1 — 0.06	0.002 ± 0.004	0.500	30	0.6	1.2 ± 0.7	1.714 27 0.1
0.008	0.26 ± 0.08	3.125	30	0.01 — 0.001	0.005 ± 0.003	1.667	30	0.2 — 0.1	1.6 ± 0.7	2.286 30 0.05 — 0.02
0.02	0.27 ± 0.08	3.375	28	0.01 — 0.001	— 0.005 ± 0.004	1.250	28	0.3 — 0.2	0.8 ± 0.7	1.143 28 0.3 — 0.2
0.2	0.29 ± 0.09	3.222	29	0.01 — 0.001	— 0.012 ± 0.004	3.000	29	0.01 — 0.001	0.2 ± 0.7	0.286 29 0.8 — 0.7

Fig. 55. Table. Normal mice. Females. 24 hrs. fasting. No food after insulin injection at 4 a. m. Decapitation at 8 a. m.

Dose	No. of animals	Body weight gm $\bar{x} \pm \epsilon_{\bar{x}}$	Liver weight gm $\bar{x} \pm \epsilon_{\bar{x}}$	Alim. canal and spleen gm $\bar{x} \pm \epsilon_{\bar{x}}$	Liver glycogen per cent $\bar{x} \pm \epsilon_{\bar{x}}$	Body glycogen per cent $\bar{x} \pm \epsilon_{\bar{x}}$	Glycogen content in mym per 10 mg of body weight minus alim. canal and spleen $\bar{x} \pm \epsilon_{\bar{x}}$
Controls	12	18.6 ± 0.6	920 ± 38	2.373 ± 87	0.62 ± 0.07	0.042 ± 0.003	7.6 ± 0.6
0.004	10	19.1 ± 0.6	880 ± 31	2.324 ± 65	0.53 ± 0.10	0.044 ± 0.003	6.9 ± 0.6
0.008	12	18.8 ± 0.5	927 ± 31	2.392 ± 83	0.69 ± 0.09	0.041 ± 0.002	7.8 ± 0.4
0.02	12	17.5 ± 0.7	867 ± 32	2.376 ± 65	0.65 ± 0.11	0.047 ± 0.002	8.6 ± 0.9
0.2	12	18.1 ± 0.6	883 ± 47	2.250 ± 130	0.32 ± 0.03	0.051 ± 0.002	6.5 ± 0.3

Fig. 56. Table showing the difference in glycogen content between controls and insulinized animals tabulated in Fig. 55.

Dose	Liver glycogen				Body glycogen				Glycogen content in mym per 10 gm of body weight minus alim. canal and spleen			
	d ± ε_d	t	df	P	d ± ε_d	t	df	P	d ± ε_d	t	df	P
0.004	0.09 ± 0.12	0.750	20	0.5 — 0.4	-0.002 ± 0.004	0.500	20	0.7 — 0.6	0.7 ± 0.8	0.875	20	0.4 — 0.3
0.008	-0.07 ± 0.11	0.686	22	0.6 — 0.5	0.001 ± 0.004	0.250	22	0.8	-0.2 ± 0.7	0.286	22	0.8 — 0.7
0.02	-0.03 ± 0.13	0.230	22	0.9 — 0.8	-0.005 ± 0.004	1.250	22	0.3 — 0.2	-0.9 ± 1.1	0.818	22	0.5 — 0.4
0.2	0.30 ± 0.08	3.750	22	0.01 — 0.001	-0.009 ± 0.004	2.250	22	0.05 — 0.02	1.1 ± 0.7	1.571	22	0.2 — 0.1

*Fig. 57. Table. Normal mice. Females. 24 hrs. fasting. No food after insulin injection at 12 p. m. Decapitation at 8 a. m.*

Dose	No. of animals	Body weight gm		Liver weight mgm		Alim. canal and spleen mgm		Liver glycogen per cent		Body glycogen per cent		Glycogen content in mgm per 10 gm of body weight minus alim. canal and spleen $\bar{X} \pm \epsilon_{\bar{X}}$	
		$\bar{X}$	$\epsilon_{\bar{X}}$	$\bar{X}$	$\epsilon_{\bar{X}}$	$\bar{X}$	$\epsilon_{\bar{X}}$	$\bar{X}$	$\epsilon_{\bar{X}}$	$\bar{X}$	$\epsilon_{\bar{X}}$	$\bar{X}$	$\epsilon_{\bar{X}}$
Controls	12	17.4	0.4	753	27	2153	68	0.74	0.06	0.062	0.004	8.5	0.4
0.004	12	16.4	0.5	758	31	2040	66	0.58	0.06	0.051	0.003	7.9	0.4
0.008	12	17.4	0.3	787	38	2027	64	0.59	0.03	0.047	0.003	7.6	0.5
0.02	12	17.1	0.6	768	45	2152	112	0.63	0.02	0.049	0.008	7.8	0.3
0.2	12	18.0	0.6	813	37	2080	85	0.79	0.11	0.062	0.005	10.0	0.8

*Fig. 58. Table showing the difference between controls and insulinized animals tabulated in Fig. 57.*

Dose	Liver glycogen				Body glycogen				Glycogen content in mgm per 10 gm of body weight minus alim. canal and spleen $\bar{X} \pm \epsilon_{\bar{X}}$									
	$d$	$\epsilon_d$	$t$	$df$	$d$	$\epsilon_d$	$t$	$df$	$d$	$\epsilon_d$	$t$	$df$	$P$					
0.004	0.116	0.08	2.000	22	0.1	0.05	0.001	0.006	0.200	22	0.9	0.8	0.6	0.6	1.000	22	0.4	0.3
0.008	0.115	0.07	2.143	22	0.05	0.02	0.005	0.005	1.000	22	0.4	0.3	1.0	0.6	1.666	22	0.2	0.1
0.02	0.111	0.06	1.833	22	0.1	0.06	0.003	0.006	0.600	22	0.6	0.5	0.7	0.5	1.400	22	0.2	0.1
0.2	-0.05	0.18	0.386	22	0.7	-0.010	0.006	1.666	22	0.2	-0.1	-1.6	0.9	1.667	22	0.2	0.1	

two hours after the injection, when the reduction is maximal as regards all the dosages. Four hours after the injection we find a statistically significant reduction only as regards the largest dosage, whilst the mice which had received the other dosages now show a good agreement with the controls. Eight hours after the injection only the animals which had received the largest dosage show a significant reduction of the liver glycogen content.

*Variations in the body glycogen content:* As shown by the tables, we find in the mice which had received 0.2 U. an increase in the body glycogen content which is statistically very probable or certain two hours after the injection, and must be regarded as probable four hours after the injection ( $P = 0.05 - 0.02$ ). As appears from the diagram, the tendency is the same one hour after the injection, and still remains so after eight hours. As regards these two latter points, however, the increase is not statistically certain in the present material. The reaction to the smaller dosages is less uniform and the values for the body glycogen content in the insulinized animals show throughout good correspondence with those of the controls.

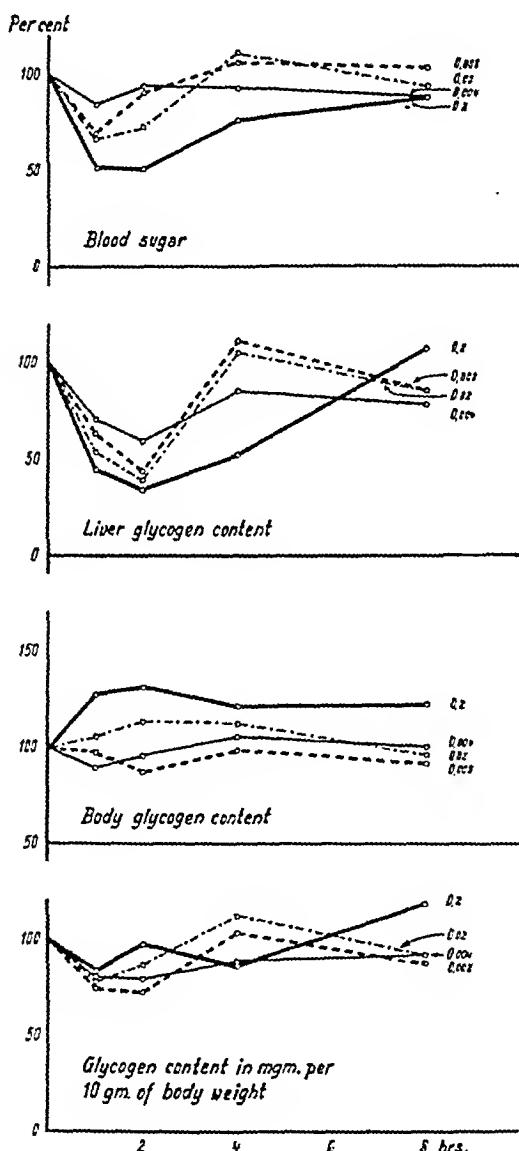
*Variations in the total amount of glycogen:* As regards the glycogen content per weight unit, we always find a good correspondence between the insulinized animals and the controls, except in one respect: the mice which had received 0.008 U. show 2 hours after the injection a reduction as compared with the controls ( $P = 0.05 - 0.02$ ).

*Variations in the blood sugar:* As regards all the dosages we find a diminution of the blood sugar, most marked 1—2 hours after the injection. The blood sugar then again rises, gradually approaching the normal value. The diminution is greater and continues longer, the larger the dosage (cf. the diagram Fig. 59).

*Recapitulation:* In this experimental group the effect of insulin on the general condition of the animals and on the blood sugar level is proportional to the dosage. It sets in more rapidly and continues longer, the larger the dosage.

All the insulin dosages tend to lower the liver glycogen content. As regards the variation in the body glycogen content, we find an essential difference in effect between the largest dosage and the

*Fig. 59. Diagrams showing the effect of increasing insulin doses on blood-sugar and storage of glycogen in mice at different times after injection. After 24 hrs. fasting insulin injection, then food. Decapitated at 8 a. m. All values given in per cent of the corresponding values of the controls.*



smaller ones, in that the latter do not result in any certain change in the body glycogen content, whereas the dosage 0.2 U. does.

**Group 3.** The results are tabulated in Figs. 60—65 and are illustrated in the diagram Figs. 66 and 67.

All the animals appeared to be unaffected.

*Variations in the liver glycogen:* The control animals, which after a 24 hours' fast, were given free access to food, showed during the first 8 hours' a very substantial continuous increase of the liver glycogen content. If they are compared with those which were decapitated at 8 a. m. after a fast of 24 hours, it will be found that the liver glycogen content has increased 2.5 times in one hour and 7 times in four hours. If the controls are compared with the animals which had received the different insulin dosages, we nowhere find any statistically significant difference. As regards the animals which had received the dosages 1 and 20 U., one hour after the injection there is possibly a slight decrease as compared with the controls ( $P = 0.05 - 0.02$  and  $0.05$  respectively).

If we study the diagrams in Figs. 66 and 67, it will be found that the animals which had received the larger insulin dosages 0.2—20 U., one hour after the injection showed a tendency to diminution of the liver glycogen content which, in magnitude and duration, was proportional to the dose.

*Variations in the body glycogen content:* In the controls, the body glycogen content shows a marked continuous increase when the animals after 24 hours fasting are given free access to food. Those which had received the smaller insulin dosages show throughout good correspondence with the controls. Those which had received 0.2—20 U. show 8 hours after the injection an increase in the body glycogen content as compared with the normal animals. In those which had received 0.2 U. insulin, the increase is so marked that it is statistically probable ( $P = 0.05 - 0.02$ ). In those which had received 1 U. it is very probable or certain, and in those which had received 20 U. it is statistically significant. At other times after the injection the insulized animals show, statistically, a good correspondence with the controls. From the diagram, however, we already see a tendency to an increase in the body glycogen content 4 hours after the injection of the two largest doses.

*Fig. 60. Table. Normal mice. Females. 2½ hrs. fasting. Food ad lib. after insulin injection at 7 a. m.  
Decapitated at 8 a. m.*

Dose	No. of animals	Body weight gm. $\bar{X} \pm \varepsilon_{\bar{X}}$	Liver weight mgm. $\bar{X} \pm \varepsilon_{\bar{X}}$	Ald. canal and spleen mgm. $\bar{X} \pm \varepsilon_{\bar{X}}$	Liver glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$	Body glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$	Glycogen content in mgm. per 10 gm. of body weight minus alim. $\bar{X} \pm \varepsilon_{\bar{X}}$
							(n = 18)
Controls	19	18.9 ± 0.3	855 ± 52	2 498 ± 100	1.72 ± 0.16	0.121 ± 0.011	20.1 ± 1.9
0.004	19	19.2 ± 0.3	851 ± 56	2 478 ± 142	1.86 ± 0.14	0.112 ± 0.011	19.9 ± 1.7
Controls	12	19.1 ± 0.8	940 ± 58	2 823 ± 172	1.71 ± 0.16	0.133 ± 0.008	22.4 ± 1.5
0.02	11	17.4 ± 0.6	775 ± 28	2 403 ± 102	1.94 ± 0.18	0.121 ± 0.016	21.5 ± 2.2
0.2	12	18.6 ± 0.6	928 ± 49	2 750 ± 150	1.36 ± 0.18	0.128 ± 0.014	20.0 ± 2.2
1	12	18.6 ± 0.3	858 ± 48	2 302 ± 81	1.17 ± 0.17	0.128 ± 0.014	18.4 ± 2.1
20	12	18.1 ± 0.5	767 ± 37	2 196 ± 60	1.17 ± 0.20	0.126 ± 0.016	17.8 ± 2.1

Fig. 61. Table showing the difference between controls and insulinized animals tabulated in Fig. 60.

Dose	Liver glycogen				Body glycogen				Glycogen content in gm per 10 gm of body weight minus adim. esophagus and spleen			
	d ± ε_d	t	df	P	d ± ε_d	t	df	P	d ± ε_d	t	df	P
0.004	-0.14 ± 0.21	0.667	35	0.6 — 0.5	0.009 ± 0.017	0.529	36	0.6	0.2 ± 2.6	0.800	35	0.5 — 0.4
0.02	-0.23 ± 0.24	0.958	21	0.4 — 0.3	0.012 ± 0.018	0.667	21	0.6 — 0.5	0.9 ± 2.7	0.333	21	0.8 — 0.7
0.2	0.86 ± 0.24	1.458	22	0.2 — 0.1	0.005 ± 0.016	0.313	22	0.8 — 0.7	2.4 ± 2.7	0.889	22	0.4 — 0.3
1	0.54 ± 0.23	2.848	22	0.05 — 0.02	0.006 ± 0.016	0.313	22	0.8 — 0.7	4.0 ± 2.6	1.588	22	0.2 — 0.1
20	0.54 ± 0.26	2.077	22	0.06	0.008 ± 0.017	0.471	22	0.7 — 0.6	4.6 ± 2.6	1.769	22	0.1 — 0.05

Fig. 62. Table. Normal mice. Females. 24 hrs. fasting. Food ad lib. after insulin injection at 4 a. m.  
Decapitated at 8 a. m.

Dose	No. of animals	Body weight gm $\bar{X} \pm \epsilon_{\bar{X}}$	Liver weight mgm $\bar{X} \pm \epsilon_{\bar{X}}$	Altn. canal and spleen mgm $\bar{X} \pm \epsilon_{\bar{X}}$	Liver glycogen per cent $\bar{X} \pm \epsilon_{\bar{X}}$	Body glycogen per cent $\bar{X} \pm \epsilon_{\bar{X}}$	Glycogen content in mgm per 10 gm of body weight minus altn. canal and spleen $\bar{X} \pm \epsilon_{\bar{X}}$
Controls	16	20.2 ± 0.3	957 ± 44	3 016 ± 145	5.51 ± 0.31	0.289 ± 0.030	58.2 ± 4.1
0.001	16	19.9 ± 0.4	866 ± 24	2 782 ± 141	5.83 ± 0.37	0.280 ± 0.020	53.9 ± 2.6
0.003	16	19.3 ± 0.3	869 ± 36	2 773 ± 133	5.28 ± 0.17	0.279 ± 0.025	54.3 ± 2.5
0.02	16	19.8 ± 0.4	866 ± 32	2 676 ± 123	5.35 ± 0.24	0.286 ± 0.017	54.7 ± 3.1
Controls	11	18.9 ± 1.0	1 080 ± 46	3 299 ± 104	4.83 ± 0.59 (n = 10)	0.295 ± 0.021	61.1 ± 5.2 (n = 10)
0.2	11	19.0 ± 0.5	1 098 ± 42	3 295 ± 274	4.93 ± 0.51	0.319 ± 0.031	64.9 ± 6.4
1.0	11	18.4 ± 0.4	1 015 ± 19	3 268 ± 132	5.21 ± 0.35	0.355 ± 0.041	68.1 ± 3.7
20	11	18.0 ± 0.4	988 ± 35	2 921 ± 135	3.99 ± 0.41	0.354 ± 0.040	59.6 ± 6.3

*Fig. 63. Table showing the difference between controls and insulinized animals tabulated in Fig. 62.*

Dose	Liver glycogen					Body glycogen					Glycogen content in mgm per 10 mg of body weight minus allim. canal and spleen			
	d ± ε_d	t	df	P	d ± ε_d	t	df	P	d ± ε_d	t	df	P		
0.004	0.18 ± 0.48	0.375	30	0.8 — 0.7	0.009 ± 0.036	0.250	30	0.9 — 0.8	4.3 ± 4.8	0.896	30	0.4 — 0.3		
0.008	0.23 ± 0.35	0.657	30	0.6 — 0.5	0.010 ± 0.039	0.256	30	0.8	3.9 ± 4.8	0.813	30	0.5 — 0.4		
0.02	0.16 ± 0.39	0.410	30	0.7 — 0.6	0.003 ± 0.034	0.088	30	> 0.9	3.5 ± 5.1	0.686	30	0.5		
0.2	- 0.10 ± 0.78	0.128	19	0.9	- 0.024 ± 0.038	0.632	20	0.6 — 0.5	- 3.8 ± 8.2	0.463	19	0.7 — 0.6		
1	- 0.38 ± 0.69	0.551	20	0.6 — 0.5	- 0.060 ± 0.046	1.304	20	0.3 — 0.2	- 7.0 ± 6.4	1.094	20	0.3 — 0.2		
20	0.84 ± 0.73	1.151	20	0.3 — 0.2	- 0.059 ± 0.045	1.311	20	0.3 — 0.2	1.6 ± 8.2	0.195	20	0.9 — 0.8		

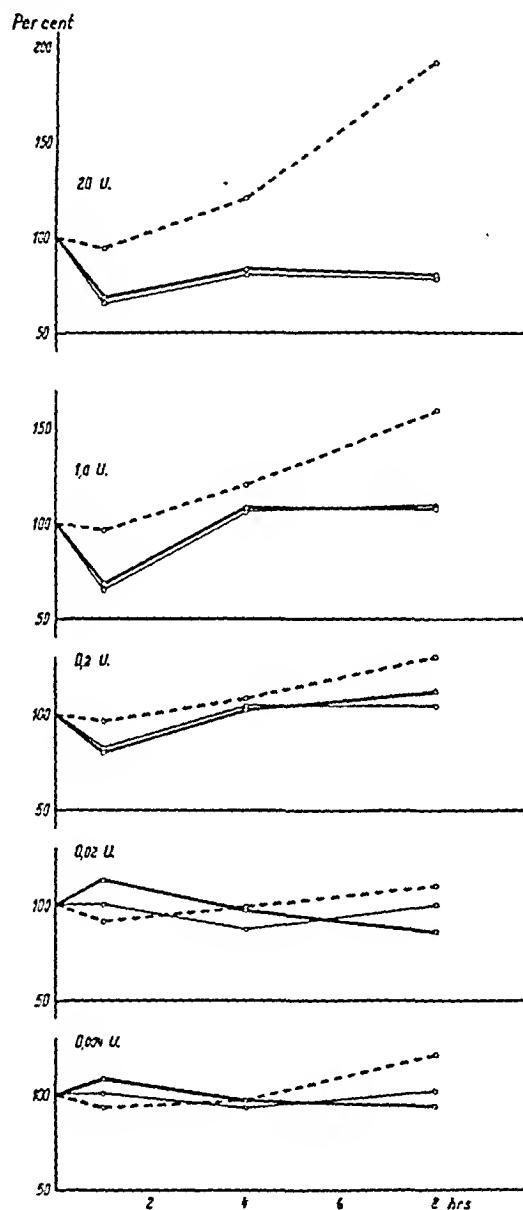
*Fig. 64. Table. Normal mice. Females. 24 hrs. fasting. Food + d lib. after insulin injection at 12 p.m.  
Decapitated at 8 a.m.*

Dose	No. of animals	Body weight gm $\bar{X} \pm \epsilon_{\bar{X}}$	Liver weight gm $\bar{X} \pm \epsilon_{\bar{X}}$	Allm. canal and spleen gm $\bar{X} \pm \epsilon_{\bar{X}}$	Liver glycogen per cent $\bar{X} \pm \epsilon_{\bar{X}}$	Body glycogen per cent $\bar{X} \pm \epsilon_{\bar{X}}$	Glycogen content in mgm per 10 gm of body weight minus alim. canal and spleen $\bar{X} \pm \epsilon_{\bar{X}}$
Controls	13	20.3 ± 0.6	1.054 ± 0.22	2.926 ± 15.9	7.27 ± 0.41	0.324 ± 0.035	75.2 ± 5.4
0.004	11	19.4 ± 0.3	0.953 ± 0.20	2.735 ± 16.7	6.84 ± 0.42 (n = 10)	0.302 ± 0.045	78.3 ± 6.5 (n = 10)
0.008	9	19.8 ± 0.6	0.956 ± 0.36	3.083 ± 20.3	6.43 ± 0.37	0.330 ± 0.035	70.2 ± 5.1
0.02	11	20.0 ± 0.3	0.930 ± 0.27	3.025 ± 19.1	6.23 ± 0.77	0.356 ± 0.050	68.3 ± 8.9
Controls	12	18.2 ± 0.4	1.055 ± 0.67	2.792 ± 4.1	5.01 ± 0.42	0.177 ± 0.015	50.5 ± 4.1
0.2	12	18.7 ± 0.4	0.988 ± 0.24	2.972 ± 16.1	5.63 ± 0.37	0.230 ± 0.016	57.2 ± 3.7
1	12	18.0 ± 0.4	0.988 ± 0.31	2.801 ± 8.5	5.42 ± 0.37	0.281 ± 0.023	61.2 ± 3.3
20	12	18.0 ± 0.5	0.903 ± 0.35	2.730 ± 13.3	4.00 ± 0.48	0.338 ± 0.025	56.4 ± 5.5

*Fig. 65. Table showing the difference between controls and insulinized animals tabulated in Fig. 64.*

Dose	Liver glycogen					Body glycogen					Glycogen content in mgm per 10 gm of body weight minus liver, canal and spleen				
	d ± ε_d	t	df	P	d ± ε_d	t	df	P	d ± ε_d	t	df	P	t	df	P
0.004	0.43 ± 0.59	0.729	21	0.5 — 0.4	-0.068 ± 0.057	1.193	22	0.3 — 0.2	-3.1 ± 8.4	0.369	21	0.8 — 0.7			
0.008	0.84 ± 0.55	1.527	20	0.2 — 0.1	-0.006 ± 0.049	0.122	20	> 0.9	5.0 ± 7.4	0.576	20	0.6 — 0.5			
0.02	1.04 ± 0.87	1.195	22	0.3 — 0.2	-0.032 ± 0.061	0.525	22	0.7 — 0.6	6.9 ± 10.4	0.663	22	0.6 — 0.5			
0.2	-0.62 ± 0.56	1.107	22	0.3 — 0.2	-0.053 ± 0.022	2.409	22	0.05 — 0.02	-6.7 ± 5.5	1.218	22	0.3 — 0.2			
1	-0.41 ± 0.56	0.732	22	0.5 — 0.4	-0.104 ± 0.028	3.714	22	0.01 — 0.001	-10.7 ± 5.3	2.019	22	0.1 — 0.05			
20	1.01 ± 0.64	1.578	22	0.2 — 0.1	-0.161 ± 0.029	5.552	22	< 0.001	-5.9 ± 6.8	0.868	22	0.4			

*Fig. 66. Diagrams showing the effect of insulin on blood-sugar and storage of glycogen in mice. Insulin injection after 24 hrs. fasting; then food ad lib. All values given in per cent of the corresponding values of the controls, which after 24 hrs. fasting were given their food simultaneously with the insulin animals. Decapitated at 8 a. m.*



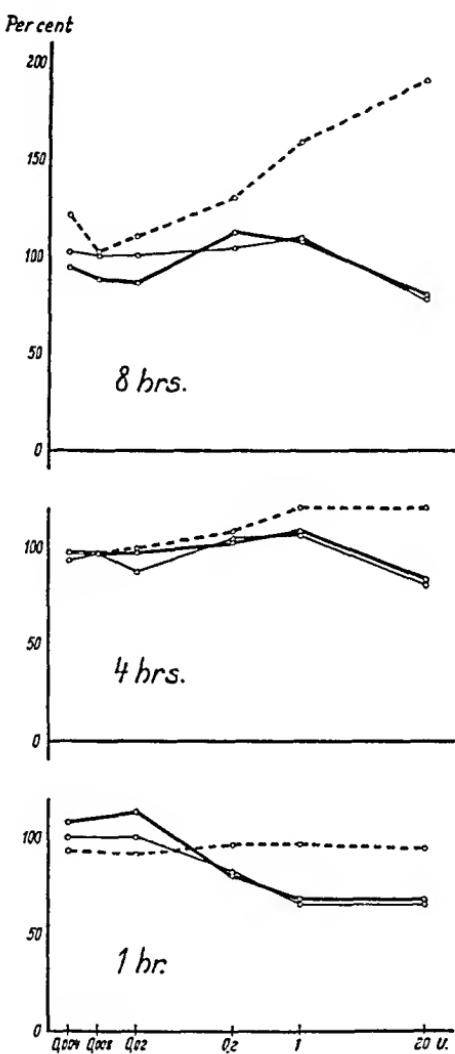
— Blood sugar.

— Liver glycogen content.

- - - Body glycogen content.

*Fig. 67. Diagrams showing the effect of time on the effect of increasing doses of insulin on blood sugar and glycogen storage in mice.*

*The same experiments as given in Fig. 66.*



— Blood sugar.

— Liver glycogen content.

.... Body glycogen content.

*Variations in the total amount of glycogen:* The amount of glycogen per weight unit in the insulinized animals shows throughout good correspondence with the controls.

*Variations in the blood sugar:* As regards the dosages 0.2—20 U., we find a decrease of the blood sugar 1 hour after the injection. As for the two smaller dosages, the blood sugar then rises to the normal value, whilst as regards the largest dosage it remains low during the whole period of observation. It is remarkable that the blood sugar curve and the liver glycogen curve always run parallel when there is a marked change in the liver glycogen content (cf. Fig. 66 and 67).

*Recapitulation.* Mice which after 24 hours fasting are given free access to food show during the next 8 hours a substantial continuous increase in the glycogen stores. The supply of small amounts of insulin has no effect on this process. The supply of larger amounts of insulin promotes the storage of glycogen elsewhere than in the liver, and the largest insulin dosage tends to reduce the storage of liver glycogen. It is noteworthy that the diminution in the storage of liver glycogen runs parallel with a sinking of the blood sugar level.

**Group 4.** It has been stated in several quarters that the qualitative effect of insulin on the glycogen stores is due to the amount of the dosage, and that by varying the latter it would be possible to obtain an increase or decrease in the glycogen content of the liver. Investigators have used the terms »physiological« and »non-physiological« dosages. By »physiological dosage« they apparently mean a dosage which does not lower the blood sugar level (FRANK, NOTHMAN and HARTMANN, 1925 and 1927, STAUB, 1930, and others).

In the experiments in group 3, no change in the blood sugar level is found after the smaller insulin doses (cf. the diagram in Fig. 66). Thus, though an effective insulin dosage had been given to these animals while the blood sugar level was high, no storage of glycogen beyond that of the controls was obtained.

As these tests, however, were not entirely comparable with those of the above-mentioned authors, further investigations were instituted. They were made on mice which had received injections

Fig. 68. Table. Normal mice. Females. Food ad lib. No food after insulin injection at 7 a. m. Decapitation at 8 a. m.

Dose to N	Body weight gm $\bar{X} \pm \epsilon_{\bar{X}}$	Liver weight mgm $\bar{X} \pm \epsilon_{\bar{X}}$	Alm. canal and spleen mgm $\bar{X} \pm \epsilon_{\bar{X}}$	Liver glycogen per cent $\bar{X} \pm \epsilon_{\bar{X}}$		Body glycogen per cent $\bar{X} \pm \epsilon_{\bar{X}}$	Glycogen content in mgm per 10 gm of body weight minus alim. canal and spleen $\bar{X} \pm \epsilon_{\bar{X}}$	Blood sugar per cent $\bar{X} \pm \epsilon_{\bar{X}}$	$\bar{X} \pm \epsilon_{\bar{X}}$ $n = 10$
				d ± $\epsilon_d$	t	df	P		
Controls	12	21.3 ± 0.5	938 ± 39	2.093 ± 58	1.65 ± 0.24	0.051 ± 0.004	13.1 ± 1.5	0.133 ± 0.005	
0.001	12	21.7 ± 0.3	1.014 ± 38	2.193 ± 59	1.52 ± 0.17	0.039 ± 0.005	11.1 ± 0.7	0.134 ± 0.008	
0.002	12	22.0 ± 0.2	985 ± 43	2.159 ± 42	1.25 ± 0.20	0.048 ± 0.004	10.7 ± 1.0	0.121 ± 0.008	

Fig. 69. Table showing the difference between controls and insulinized animals tabulated in Fig. 68.

Dose	Liver glycogen						Body glycogen				d ± $\epsilon_d$ df t P					
	d ± $\epsilon_d$	t	df	P	d ± $\epsilon_d$	t	df	P	d ± $\epsilon_d$	df						
0.001	0.13 ± 0.29	0.448	22	0.7	0.012 ± 0.0064	1.875	22	0.1 — 0.05	2.0 ± 1.7	1.176	22	0.3 — 0.2				
0.002	0.40 ± 0.31	1.290	22	0.3 — 0.2	0.003 ± 0.006	0.500	22	0.7 — 0.6	2.4 ± 1.8	1.333	22	0.2 — 0.1				

Fig. 70. Table. Normal mice. Females. 24 hrs. fasting. Subcutaneous saline injection at 7 a. m. No food. Decapitation at 8 a. m.

Dose	No. of animals	Liver glycogen per cent	Body glycogen per cent	Glycogen content in mgm per 10 gm body weight minus alim. canal and spleen	Blood sugar per cent
Controls	12	0.32 ± 0.04	0.044 ± 0.008	5.5 ± 0.4	0.088 ± 0.002 (n = 8)
NaCl	12	0.37 ± 0.03	0.040 ± 0.002	5.6 ± 0.3	0.086 ± 0.002 (n = 10)
d ± ε_d		0.05 ± 0.05	0.004 ± 0.004	0.1 ± 0.5	

of 0.001 and 0.002 U. without previous fasting. After the injection they were left without food until the decapitation at 8 a. m., one hour after the injection. The results are tabulated in Fig. 68 and 69. As regards none of these dosages could any change in the glycogen content be observed and the smallest dosage, as was ascertained, did not entail any change in the blood sugar level.

It is, of course, conceivable that in the case of these small insulin dosages, the irritation involved in the actual injection might entail changes in the glycogen stores which might be mistaken for the effect of the insulin. True that the well-known »Fesselungshyperglykämie« was avoided, but it has been shown that emotional excitation may cause an increase in the blood sugar (for the literature, see HOLMGREN and WOHLFAHRT 1944). As MANN and his co-workers have convincingly shown that we need not reckon with other sources of the blood sugar than the liver glycogen, it must be concluded that a change in the liver glycogen content had actually occurred, unless, concurrently with the rise in the blood sugar, the injection had stimulated the glycogenesis of the liver. SILVETTE and BRITTON (1932), in connection with a study of the glycogen storage, state: »Profound emotional excitation for a brief period also brings about change in carbohydrate values essentially similar to those observed after severe exercise.»

In order to settle this matter, I arranged serial tests with fasting mice. I injected a corresponding amount of physiological saline solution subcutaneously and after one hour analyzed the glycogen content in the mice. They were then compared with a concurrently analyzed control series of fasting mice. The results are tabulated in Fig. 70. It will be seen from the table that the two series show good correspondence throughout, which indicates that the injection as such had no effect on the amount of the glycogen stores.

*Recapitulation.* These experiments show that with very small insulin doses, if they affect the liver glycogen content at all, always gives a reduction. There was no indication in support of the view that insulin in very small doses, so small that they do not affect the blood sugar level, brings about an increase in the liver glycogen content.

## Experiments on rats

Very thorough studies on the effect of insulin on the glycogen stores of rats have been made by CORI and CORI. These studies were as a rule made on a large material and the results are convincing. On most points they correspond with my experiments on mice. For this reason merely a few experiments were made with rats, namely where the results diverge and in a few other interesting cases.

**Group I.** According to CORI and CORI (1929) insulin does not affect the liver glycogen content in rats which receive the injection after a fast of 24—48 hours. Under these conditions the muscle glycogen is reduced. As these results differ considerably from my own observations in the experiments on mice, a control investigation was made. After a fast of 24 hours the rats received insulin subcutaneously, were afterwards fasted for two more hours, after which they were decapitated and analyzed. The results are tabulated in Fig. 71 and 72. As the results show, CORI and CORI's statements that the liver glycogen under these conditions is not affected by the supply of insulin were completely confirmed. Also as regards the muscle glycogen, my results correspond with the figures given by CORI and CORI. As regards the smaller dosage there is no change, but for the larger one a statistically probable reduction ( $P = 0.05 — 0.02$ ). We are concerned here, however, with very small figures. Moreover, most of the animals which received the larger dose of insulin had convulsions, for which reason no importance can be attached to the reduction of the muscle glycogen content. The glycogen content of the skin in these experiments shows no marked variations. The insulin dosages were so adjusted that the smaller ones had left the animals completely unaffected.

Fig. 71. Table. Normal rats. 24 hrs. fasting. No food after insulin injection at 3 p. m. Decapitation at 5 p. m.

Dose	No. of animals	Body weight gm $\bar{X} \pm \varepsilon_{\bar{X}}$	Liver weight gm $\bar{X} \pm \varepsilon_{\bar{X}}$	Liver glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$	Muscle glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$	Skin glycogen per cent $\bar{X} \pm \varepsilon_{\bar{X}}$	Blood sugar per cent $\bar{X}$
Controls	11	149 ± 6	4.7 ± 0.3	0.19 ± 0.03	0.31 ± 0.02	0.15 ± 0.01 (n = 9)	0.085
0.1	12	149 ± 8	4.7 ± 0.3	0.18 ± 0.02	0.28 ± 0.03	0.17 ± 0.01 (n = 9)	0.077
1	12	144 ± 6	4.5 ± 0.2	0.17 ± 0.01	0.24 ± 0.02	0.15 ± 0.01 (n = 9)	0.048

Fig. 72. Table showing the difference between controls and insulinized animals tabulated in Fig. 71.

Dose	Liver glycogen						Muscle glycogen						Skin glycogen					
	$d \pm \varepsilon_d$	t	df	P	$d \pm \varepsilon_d$	t	df	P	$d \pm \varepsilon_d$	t	df	P	$d \pm \varepsilon_d$	t	df	P		
0.1	0.01 ± 0.04	0.250	21	0.9 — 0.8	0.03 ± 0.04	0.750	21	0.5 — 0.4	-0.02 ± 0.01	2.000	16	0.1 — 0.05						
1	0.02 ± 0.03	0.667	21	0.6 — 0.5	0.07 ± 0.03	2.333	23	0.05 — 0.02	0	-	16	-						

Fig. 73. Table. Normal rats, 24 hrs. fasting. Food ad lib. after insulin injection at 1 p. m. Decapitation at 5 p. m.

Dose	No. of animals	Body weight gm		Liver weight gm		Liver glycogen per cent		Muscle glycogen per cent		Skin glycogen per cent		Blood sugar per cent $\bar{X} \pm \epsilon_{\bar{X}}$
		$\bar{X}$	$\epsilon_{\bar{X}}$	$\bar{X}$	$\epsilon_{\bar{X}}$	$\bar{X}$	$\epsilon_{\bar{X}}$	$\bar{X}$	$\epsilon_{\bar{X}}$	$\bar{X}$	$\epsilon_{\bar{X}}$	
Controls	12	148	13	5.8	0.6	3.72	0.30	0.78	0.06	0.26	0.01	0.119
0.1	12	143	10	5.6	0.4	3.39	0.34	0.87	0.06	0.34	0.06	0.121
1	12	136	10	5.1	0.4	3.18	0.28	0.80	0.06	0.29	0.02	0.123
10	12	152	10	5.7	0.3	2.47	0.38	1.00	0.04	0.44	0.07	0.093

Fig. 74. Table showing the difference in glycogen content between controls and insulinized animals tabulated in Fig. 73.

Dose	Liver glycogen						Muscle glycogen						Skin glycogen					
	$d \pm \epsilon_d$	t	df	P	$d \pm \epsilon_d$	t	df	P	$d \pm \epsilon_d$	t	df	P	$d \pm \epsilon_d$	t	df	P		
0.1	0.33 ± 0.45	0.733	22	0.5 — 0.4	-0.09 ± 0.08	1.126	22	0.3 — 0.2	-0.09 ± 0.06	1.800	22	0.1 — 0.05						
1	0.59 ± 0.41	1.439	22	0.2 — 0.1	0.02 ± 0.07	0.286	22	0.8 — 0.7	-0.04 ± 0.02	2.000	22	0.1 — 0.05						
10	1.25 ± 0.48	2.604	22	0.02 — 0.01	-0.22 ± 0.07	3.143	22	0.01 — 0.001	-0.19 ± 0.07	2.714	22	0.02 — 0.01						

at the time of decapitation, whilst the larger doses at the time of decapitation had greatly affected half of the animals and had slightly affected the other half.

**Group 2.** In these experiments, the rats after a 24 hours fast were given an insulin injection and afterwards free access to food. In addition to the liver and muscle glycogen, the glycogen contents of the skin was also determined, in order to control statements made by previous authors such as SCOTT (1929) and RICHTER (1931), who stated that insulin under these conditions had not affected the storage of glycogen in the fatty tissue and the skin. The results are tabulated in Fig. 73 and 74. After the two smallest dosages 0.1 and 1 U. per 100 gm of body weight the blood sugar level remains unchanged. But after the largest dosage 10 U. to 100 gm, the fall in the blood sugar is slight, but noticeable. As regards the smaller dosages, all the glycogen analyses show good correspondence between the insulinized animals and the controls. As regards the largest insulin dosage we find a statistically probable reduction of the liver glycogen content, an almost certain increase of the muscle glycogen content and a probable increase of the skin glycogen content ( $P = 0.02 - 0.01$ ). These investigations show that insulin under the experimental condition in question does not stimulate the storage of glycogen in the liver and that large doses counteract it. These results correspond to CORI and CORI's investigations into the effect of insulin during «the absorptive state». As regards the muscle glycogen, the glycogen storage is stimulated by large doses of insulin.

### Summary.

In complete agreement with CORI and CORI, insulin under the experimental conditions in question cannot be shown to have any effect on the liver glycogen content of fasting animals which receive no food after the injection. The smaller dose does not seem to entail any change in the muscle glycogen content.

In fasting animals which receive food after the injection the large insulin dosage entails a reduction of the liver glycogen storage and an increase of the glycogen storage in muscles and skin.

## Experiments on rabbits

The most confusing and contradictory statements regarding the effects of insulin are found in that part of the literature which deals with investigations on rabbits (cf. the survey of the literature in Fig. 38). It has therefore seemed to be necessary to investigate the effect of insulin also on these animals.

**Group 1.** The rabbits received an injection of insulin at 3 p. m. without a preceding fast. After the injection they had no access to food. They were decapitated and analyzed after the lapse of three hours. The results are tabulated in Fig. 75. According to these investigations, insulin under these conditions has no effect on the glycogen stores of the rabbit, at any rate not with the dosages employed, 10 U. per kg of body weight subcutaneously, and at the time when the analyses were made.

It is a generally known and recognized fact that the glycogen depots in the rabbit are subject to very great variations. This was the case also in my experimental series. In this way a minor effect of the insulin might possibly be disguised. Moreover, the alimentary canal of the rabbit contains a large and very variable amount of carbohydrates, which may affect the results. In view of these

*Fig. 75. Table. Normal rabbits. Food ad lib. No food after insulin injection at 3 p. m. Decapitated at 6 p. m.*

Dose	Number of animals	Body weight gm	Liver weight gm	Liver glycogen per cent	Muscle glycogen per cent
Controls	16	1938 ± 74	74.8 ± 4.1	9.28 ± 0.82	0.88 ± 0.04
10 U/kg	16	1908 ± 106	69.7 ± 4.6	8.27 ± 0.96	0.40 ± 0.08

*Fig. 76. Table. Normal rabbits. 24 hrs. fasting. No food after insulin injection at 8 p. m. Decapitated at 5 p. m.*

Dose	Number of animals	Body weight gm	Liver weight gm	Liver glycogen per cent	Muscle glycogen per cent	Blood sugar per cent
Controls .	13	2231 ± 77	58.8 ± 7.0	1.88 ± 0.29	0.28 ± 0.08	0.092
0.2 U/kg	13	2162 ± 61	63.4 ± 3.0	1.78 ± 0.82	0.28 ± 0.08	0.088
1 U/kg .	13	2319 ± 118	59.4 ± 3.9	1.81 ± 0.47	0.28 ± 0.04	0.050

marked individual variations in the glycogen stores, the rabbit must be considered to be an unsuitable experimental object for studies of this kind.

**Group 2.** According to several authors, especially GOLDBLATT and CORKILL, an increase in the liver glycogen of fasting rabbits is found on the supply of insulin. This entirely deviates from what most investigators have shown in regard to other kinds of animals as well as from my own results in experiments on mice and rats. A control investigation therefore seemed to be indicated. In my experiments I used the dosages 0.2 and 1 U. per kg of body weight. When the animals were decapitated two hours after the injection, almost all of them seemed to be unaffected. The results of the glycogen analyses are tabulated in Fig. 76. As shown by this table, the correspondence between the insulinized animals and the controls is throughout good. It is, however, also evident that the individual variations in the liver glycogen content even in fasting animals is very marked. With a view to further control of this important question, serial experiments were arranged as follows. The rabbits after a 24 hour fast were given 0.5 U. insulin subcutaneously per kg of body weight, at 1 p. m. As soon as they had become apathetic and had difficulty in keeping an upright position, they were decapitated and analyzed. A control animal was always examined concurrently with every insulinized animal. The results are tabulated in Fig. 77. By computing the mean difference and its standard error in this experimental series, a systematic difference in the liver glycogen content will be found between insulinized animals and controls, the liver glycogen content of the

*Fig. 77. Table showing the effect of insulin on glycogen storage in normal male rabbits, 24 hours fasting. No food after subcutaneus injection of 0.5 U of insulin per kgm. of body weight at 1 p. m. Insulinized animal and corresponding control animal decapitated as soon as the insulinized animal showed flaccidity and loss of coordination.*

Time of decapitation	Insulin animals			Control animals			Liver glycogen. Mean of insulin and control animal per cent	Liver glycogen. Difference between control and insulin animal per cent	Difference in per cent of mean
	Body weight gm	Liver weight gm	Liver glycogen per cent	Body weight gm	Liver weight gm	Liver glycogen per cent			
14.25	1400	31.0	0.25	1650	38.3	0.42	0.84	0.17	50
14.30	1350	42.1	0.88	1500	42.5	0.78	0.83	-0.10	-12
14.35	1500	36.4	0.26	1700	45.2	0.37	0.32	0.11	34
14.50	1100	27.0	0.50	1400	42.2	2.99	1.75	2.49	140
15.00	1500	43.7	1.06	1750	58.2	2.82	1.94	1.76	91
15.15	1800	46.6	0.36	1800	51.5	1.80	0.83	0.94	113
15.50	1300	32.9	0.60	1500	39.0	1.22	0.91	0.62	77
16.15	1400	40.7	0.66	1650	54.8	0.88	0.77	0.22	29
16.20	1850	50.4	1.85	1900	70.9	3.71	2.78	1.86	67
16.25	1500	40.1	2.32	1750	46.7	0.90	1.61	-1.42	-91

$$\bar{d} \pm e_{\bar{d}} = 49.8 \pm 10.1$$

$$t = 4.981$$

$$df = 9$$

$$P = < 0.001$$

insulinized animals being lower than that of the controls. Thus even rabbits react by a reduction of the liver glycogen content under these experimental conditions, thereby showing no essential difference in their reaction from other laboratory animals commonly employed. It is difficult to find out the reason why authors such as GOLDBLATT and CORKILL have arrived at opposite results. A conceivable possibility is that they have not taken into consideration the source of error involved in the diurnal variations in the liver glycogen content. Whether this is actually the fact cannot, however, be inferred from the scanty data in their publications.

## **Summary.**

In view of the marked individual variations to which the glycogen storage in rabbits is subject, these animals are unsuitable for investigations of this nature.

The results of the investigation do not bear out GOLDBLATT'S and CORKILL'S view that insulin injections into rabbits bring about a storage of glycogen in the liver, but rather indicate a reduction, as in the case of other experimental animals.

## General survey of results

According to the general view, the liver and the skeletal muscles are *the principal storehouses in the body for glycogen*. Only under certain special conditions, free access to food after fasting for some length of time, can the adipose tissue and the skin also store considerable amounts of glycogen (Chapter 4).

In Chapter 5 the *distribution of glycogen within a glycogen depot* is discussed. The statements in the literature vary. The author's own investigations do not rule out a difference in glycogen content in different parts of the liver, but they argue against a systematic difference of this kind, such as that a certain lobe of the liver should regularly contain more glycogen than the other parts of the liver. As regards the muscles, we find marked variations in glycogen content between different muscles from the same animal as well as between the same muscles from different animals. A statistical analysis of the results, however, provides no basis for any systematic difference. Thus in serial analyses, a result from one muscle is considered to be representative of the entire musculature, as also a piece of liver for the whole liver.

*The mode of killing and the postmortal glycogenolysis* (Chapter 6) has also been considered to play a large part in the amount of glycogen that could be shown in the depots. It has been stated by various investigators that the samples should be taken during a rapidly induced narcosis, but, on comparison, it has been found that this method has no advantages as compared with decapitation. Nor does freezing in liquid air afford any advantages. The investigation into postmortal glycogenolysis shows that we need not reckon with it as a complicating factor if the pieces of tissue are plunged into hot KOH within two minutes after decapitation.

*The age and sex of the animals* are considered by a number of authors to affect the glycogen storage in the body (Chapter 7). The statements in the literature are based in several cases on

large serial experiments. In my own investigations, however, no sex differences could be observed. On the other hand, it appears that the age of the animals might have some bearing on the liver glycogen content. No effect on the muscle glycogen content has been observed.

The effect of the *composition of the diet and the length of the fasting period* is discussed in Chapter 8. From the literature, various investigations indicating that the composition of the diet is of great importance for the amount of the glycogen storage have been cited. The length of the fasting period is also an important factor. A summary of investigations on animals with different fasting periods shows that both the liver and the muscle glycogen content is greatly dependent on the length of the fasting period. The reduction of the glycogen content in mice culminates after about 24 hours fasting, and the glycogen content then shows a tendency to rise again.

Data from the literature regarding variations in the amount of the glycogen stores according to *the time of day, the season and the outdoor temperature* are summarized in Chapter 9. Diurnal changes must be considered to occur, likewise variations due to the temperature. On the other hand, no certain seasonal variations due solely to the season seem to have been ascertained.

In addition to these factors, which can be more or less controlled or eliminated by the experimenter, the glycogen depots are affected by other factors which are entirely beyond control (Chapter 10). These factors can be neutralized by *concurrently examining experimental animals and controls, by distributing the serial experiment over several days and by subjecting the results to statistical analysis*.

There are a large number of reports on different factors which affect the sensitivity of the individual to insulin. All these factors have proved to be the same as those which affect the storage of glycogen in normal animals.

Chapter 11 contains a short review of *the different methods of studying the effect of insulin on the glycogen depots and of the different results attained by experiments on intact animals*. As indicated by this survey, no agreement has been reached in regard to the effect of insulin on the glycogen storage. Most of

the investigations have been made merely on very few animals, and, in view of the marked individual variations, are therefore by no means convincing. But even those investigations that have been made on the basis of a larger material have yielded contradictory results. Thus, for example, FRANK, NOTHMANN and HARTMANN, GOLDBLATT and CORKILL state that insulin tends to increase the storage of glycogen in the liver, whilst CORI and CORI, LAWRENCE and McCANE, RUSSEL, BRIDGE, BÜRGER and KOHL consider that they can show a decrease. VENDÉG considers that the result depends on the amount of the dose. As regards the muscles, an increase of the glycogen content after the supply of insulin is noted by RUSSEL, CORKILL, BRIDGE, CORI and CORI, a decrease by e.g. LAWRENCE and McCANE, and by HANDOWSKY, whilst FRANK, NOTHMANN and HARTMANN as well as GOLDBLATT fail to find any change.

The experiments recorded in the literature were as a rule made with single dosages and the results were analyzed at a certain time after the injection. Merely sporadic attempts were made, for example by VENDÉG, GOLDBLATT, CORI and CORI, to compare different dosages. These comparative tests were never systematized, nor have the conditions at different times after the injection been investigated to the desirable extent.

Chapter 12 reports a systematic investigation on mice into the effect of different insulin dosages on the glycogen storage at different times after the injection and under different food conditions. On each occasion the investigations were made on several mice and the results were subjected to statistical analysis. It appears from these studies that the supply of insulin brings about a reduction of the liver glycogen content. This applies to all dosages that have any effect at all. The decrease in liver glycogen, in amount and duration, is proportional to the dose. No increase of the liver glycogen storage after the injection of insulin has ever been observed. Furthermore, the interesting fact was noted that the reduction in the storage of liver glycogen always runs parallel with a reduction in the blood sugar. The glycogen content in the rest of the body, reckoned in percentage, is increased under the action of insulin, provided that the doses are sufficiently large. In the case of smaller doses — except possibly in the experiments under

group 1—, there is no certain change. As regards *the effect of insulin on the total amount of glycogen per unit of body weight*, there is some difference under different experimental conditions. In the animals which receive an injection of insulin without previous fasting a decrease of the total amount of glycogen is found, in the others no change.

*Chapter 13* contains a report on *supplementary experiments on rats*. The results are in complete accordance with the thorough investigations previously made by CORI and CORI. These animals too — if any noticeable changes are found as the result of the insulin injection —, show a fall in the liver glycogen content and a rise in the glycogen content of the muscles and skin. The fall in the muscle glycogen content observed in fasting animals after large doses of insulin seems to be due to the hypoglycemic convulsions.

*Chapter 14* gives an account of *experiments on rabbits*. The reports in the literature in regard to the effect of insulin on rabbits are very confusing. As regards the liver, some authors report an increase of the glycogen content under the action of insulin, others a decrease. This seems to be due to the fact that the liver glycogen content in the rabbit, even under standardized experimental conditions, is subject to very marked individual variations. This, in conjunction with the fact that most investigators have examined a very small number of rabbits, seems to be the explanation of the divergent results.

The author's investigations, which were especially intended to control the statements to the effect that in young starving rabbits insulin brings about a storage of glycogen in the liver, indicate that this is not the case. Rabbits, like other experimental animals, react to a supply of insulin — if it produces any effect at all —, by a fall of the liver glycogen content. This result, of course, is exactly the reverse of that found e.g. by GOLDBLATT and CORKILL, who nevertheless worked with rather large series. The reason for this divergence is not clear, but their results may be accounted for by some overlooked source of error. It would be very peculiar if one kind of animal should react to insulin in an essentially different way from others.

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FROM THE DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF LUND, SWEDEN

ON THE PRESENCE OF HISTAMINE IN  
PLASMA IN A PHYSIOLOGICALLY  
ACTIVE FORM

BY

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## Chapter 1

### Introduction

Only a few years after the synthesis of  $\beta$ -imidazolylethylamine it was discovered that this substance has remarkable biological effects, contracting plain muscles, dilating capillaries and stimulating glandular activity (Dale and Laidlaw 1910, Popielski 1920). About the same time it was shown that this active base normally occurs in the body and seems to be an ordinary constituent of the cell (Barger and Dale 1911, Abel and Kubota 1919, Best, Dale, Dudley and Thorpe 1927). Resulting from these discoveries it was suggested that histamine was engaged in several normal and pathological processes in the body. Thus histamine is thought to play a part in the local regulation of the blood flow through the tissues, e. g. causing vasodilatation in reactive hyperaemia and in muscular activity. Further histamine has been referred to as »the hormone for gastric secretion» and has also been considered a normal stimulant of intestinal motility. Examples of pathological conditions where histamine has been thought to play a more or less important rôle are: surgical shock, shock secondary to burns, peptic ulcer, toxæmias in pregnancy, anaphylactic shock, hypersensitiveness to cold, urticaria, hay fever, Ménière's syndrome, a special type of headache, intestinal autointoxication etc. Histamine is also supposed to be responsible for some of the symptoms produced by snake venoms, bee venom and the toxins of certain bacteria. A great number of monographs and articles dealing with theories concerning the part played by histamine in these and other reactions have been published (Dale 1920, 1929, 1933, Feldberg and Schilf 1930, Küpper 1930, Best and McHenry 1931, Gaddum 1936, Guggenheim 1940, Rigler 1938, Code and MacDonald 1937,

Feldberg 1937, Gajdos 1938, Marcou et al. 1938, Ahlmark and Kornerup 1939, Dragstedt 1940, 1945, Rocha e Silva 1944).

These theories aroused interest in the estimation of histamine occurring in blood and especially in blood plasma. Although it is believed that under physiological conditions histamine acts locally as »Gewebshormon», many investigators have tried to trace the histamine instrumental in these reactions in the venous blood emerging from the tissue. Already at an early stage of investigation in this field it was believed that in pathological processes, especially in severely damaged tissue, histamine is liberated locally, absorbed into the blood stream, and carried to distant organs causing specific reactions in the periphery.

These investigations led to the discovery that histamine is a normal constituent of blood. The experiments of Harris (1927) and Best and McHenry (1930) suggested that histamine is normally present in the blood. This was, however, denied by Koessler and Hanke (1924), Guttentag (1931), Zipf (1931), Zipf and Hülsmeyer (1933), Mac Gregor and Peat (1933) and Mac Gregor and Thorpe (1933). After Barsoum and Gaddum (1935) had devised a method for the estimation of histamine — later modified by Code (1937) — the presence of histamine in normal blood must be considered to be well founded. In their first paper on this subject Barsoum and Gaddum also demonstrated the presence of histamine in plasma by this method, a finding which has been confirmed by later investigators using similar methods.

The fact that histamine is present in plasma is rather puzzling from several points of view. It is remarkable that this highly active substance can be found in plasma in relatively high concentrations. In the guinea-pig the plasma contains approximately  $200\gamma$  of histamine per litre plasma or even more. If a saline solution containing histamine in this concentration is perfused through the blood vessels or through the bronchial tree of an isolated guinea-pig's lung it elicits a marked bronchoconstriction (Bartosch, Feldberg and Nagel 1932, Epstein 1932). A piece of guinea-pig's ileum, suspended in Tyrode's solution, usually contracts strongly when histamine is added in a quantity giving a concentration of  $5\gamma$  per litre in the bath; if the concentration is increased to e. g. one fourth of the plasma concentration a

maximal contraction is likely to occur. It is also striking that minute amounts of histamine, when injected intravenously, are capable of causing large physiological effects although the plasma of the animal contains histamine in a relatively high concentration. In fact, on injection of amounts causing large physiological effects, the increase in the histamine content of the blood or plasma is so small that it can not be detected by the methods available (Rose 1940, Emmelin, Kahlson and Wicksell 1941).

It is also remarkable that histamine occurs in the plasma in spite of the presence of a histaminolytic factor in the blood. In several species where the plasma has a histaminolytic activity, the plasma gives histamine on extraction; in various instances we have found histamine in the plasma of pregnant women who are at such a period when the histaminolytic power of plasma is known to be very great (for a detailed report on the histaminolytic power of plasma see Ahlmark 1944). Not only plasma but also certain tissues contain agents which should make it impossible for histamine to be present in plasma. Best and McHenry (1930) found that a dog's kidney perfused with blood containing added histamine was, within four hours, able to destroy as much as 200 mg of histamine. A heart-lung-kidney-preparation is capable of inactivating 25 mg of histamine in 15 minutes (Steggerda, Essex and Mann 1935). A high histaminolytic power in isolated perfused organs has also been found by Mac Gregor and Peat (1933) and Sibul (1935). The well-known fact that the effect of intravenously injected histamine is very transient is partly due to the histamine inactivating power of the organism: the histamine content of the plasma rapidly decreases to its original level.

It is thus obvious that the alleged appearance of histamine in blood plasma gives rise to a series of problems. Some of the ways in which these problems may be dealt with will be considered here.

The first question arising is this: is it proved beyond doubt that the substance found in *plasma* is really histamine? It must be born in mind that the biological methods used for detection of histamine are not specific. The presence of histamine in

*blood* seems to be well established: estimations with different test objects and certain specific reactions all point in this direction; besides, using rabbit's blood, which is especially rich in histamine, Code and Ing (1937) were able to isolate and chemically identify histamine from »the white layer». This is, however, not directly applicable to the small part of the blood histamine which is claimed to be present in plasma; so far, it can only be stated that plasma, treated according to the usual methods of extraction, stimulates the isolated guinea-pig's gut and the fowl's rectal coecum. Although it seems probable that the substance in question is histamine, further attempts to identify the agent appear necessary.

A second method of treating the problems is suggested by the following facts. Barsoum and Gaddum (1935), investigating rabbit's blood, found about 15 per cent of the blood histamine in plasma. Anrep and Barsoum (1935) and Code (1937), continuing this work, found a somewhat smaller part of the blood histamine in plasma, and Minard (1941) stated, that only 2—3 per cent of the histamine is contained in the plasma. It is obvious that the more the methods have been improved, the smaller has become the part of the total blood histamine which can be found in plasma. It has been demonstrated that most of the blood histamine, at least in the rabbit, is present in the platelets (Schwartz 1936, Zon, Ceder and Crigler 1939, Minard 1941). Considering the fragility of the platelets it is reasonable to suspect that physiologically plasma does not contain any histamine at all; the small quantity of histamine found in a plasma sample might have been liberated at the breakdown of some few platelets during the collection and centrifuging of the blood samples; even the slightest tendency of clotting will have the same effect. Particularly where the rabbit is concerned this interpretation seems reasonable as the platelets of this animal are exceptionally rich in histamine. Minard (1941) suggests such a possibility. With reference to human blood this possibility has, under somewhat different aspects, been discussed by Barsoum and Smirk (1936).

A third possibility is, that histamine is really contained in the plasma but is present there in such a form that it is

physiologically inactive and protected against the histaminolytic effect of blood and tissues. It should be emphasized that the biological estimation of the histamine content of plasma is proceeded by drastic chemical extraction processes. Gaddum (1936) points out that the demonstration of histamine in blood in this way »would not justify the view that histamine is present in a free and active form . . . These extracts are subjected to prolonged boiling in the presence of strong acid. . . . one of its effects may be to liberate histamine from an inactive precursor. The evidence on this latter point is still incomplete.» Also Dale (1937, 1938) and Feldberg (1937) have emphasized that the available methods do not answer the question whether histamine is present in blood in a free and active form or in a combined, inactive state.

The following investigation is an attempt to treat these problems from the three points of view which have been discussed above.

## Chapter 2

# Method of quantitative estimation of histamine in blood plasma

In these experiments we have used plasma from guinea-pigs, rats, rabbits, cats and dogs. The chemical methods available are obviously not sensitive enough for the detection of the small amounts of histamine in plasma. In this chapter we will only refer to the guinea-pig gut method, which was used as a routine. Other test objects have also been used, but as this has been done chiefly to identify the histamine they will be discussed in chapter 3. The method for estimation of histamine in plasma with the guinea-pig's ileum as a test object, such as is practised in this laboratory, has been described previously (Emmelin, Kahlson and Wicksell 1941) and a few details only will be added here.

*Assay on isolated guinea-pig's gut.* Zadina (1939) stated that the gut should be taken from young guinea-pigs, weighing 200—250 g. Like Ahlmark (1944) we have come to the conclusion, that it is preferable to use larger animals; the gut of animals weighing more than 300 g is more sensitive to histamine and it also gives constant responses to the same dose of histamine for a much longer period. It is often claimed that the animal should be starved for some time before the assay; our opinion is that this is not of any importance.

In several experiments we have worked on preserved gut. Guggenheim and Löffler (1916), who introduced the guinea-pig gut as test object for histamine, suggested such a method, later recommended by Minard (1941).

After the guinea-pig has been killed by a blow on the head an empty piece of gut is removed. The gut is kept in Tyrode solution in the refrigerator.  $1\frac{1}{2}$ —2 hours before the assay a small piece is put into warm Tyrode solution, through which oxygen is bubbled. The gut

soon begins to contract spontaneously and can then be used in the ordinary way. Pieces of the same gut, preserved in this manner, may often be used 4—5 days after the animal has been killed.

The gut is suspended in a bath containing about 2 ml of Tyrode solution. Oxygen holding 1 per cent of carbon dioxide is blown through the bath.

The Tyrode solution is prepared in the following way. To 40 ml of solution I and 20 ml of solution II aq. dest. ad 1000 ml is added. Solution I: NaCl 200 g, KCl 5 g, CaCl<sub>2</sub> + 6H<sub>2</sub>O 10 g, MgCl<sub>2</sub> + 6H<sub>2</sub>O 5 g, aq. dest. ad 1000 ml. Solution II: NaH<sub>2</sub>PO<sub>4</sub> 1.25 g, aq. dest. ad 500 ml. This solution also contains NaHCO<sub>3</sub> in a quantity, which is determined each time new base solutions are prepared; this quantity is chosen so as to give the Tyrode solution when thoroughly bubbled with oxygen holding 1 per cent CO<sub>2</sub>, a pH of 7.1—7.3 (determined electrometrically).

Kwiatkowski (1941), testing histamine on guinea-pig's gut with a special perfusion method, described by Gaddum, Jang and Kwiatkowski (1939), suggested a Tyrode solution with only one half of the usual amount of CaCl<sub>2</sub>. In this way the sensitivity of the gut to histamine was greatly increased. McDowall and McWhan (1936) have also noticed that histamine effects are more pronounced in a solution poor in calcium. We have tried this solution in the usual test system but abandoned it since we have found that the assay is rendered more difficult by the impaired relaxation of the gut. Addition of glucose to the Tyrode solution, also recommended by Kwiatkowski, increased neither the sensitivity nor viability of the gut in our experiments.

In some experiments the Tyrode solution has contained atropine sulphate in the concentration of 1:2 millions, but we are not convinced that this is of any advantage when examining blood plasma; surely it greatly reduces the sensitivity of the gut to histamine.

In the biological assay the plasma samples have been matched against a standard solution of histamine biphosphate, containing 100 γ of histamine base per litre. This solution has been freshly prepared before each experiment. All values in this paper are given in terms of histamine base.

*Preparation of the plasma for biological assay.* Before the estimation on the gut takes place the plasma samples must be treated by a method, which leaves as much as possible of the histamine intact but eliminates substances which interfere with the biological assay. Two methods have been used. Firstly the plasma has been treated according to the chemical method of Barsoum and Gaddum (1935) as modified by Code (1937). Code's method has the advantage of being simpler and of not

causing so great a loss in histamine as the original one (Code 1937, Code, Evans and Gregory 1938). Anrep et al. (1939) claim that the Code method gives higher values when used on blood than the Barsoum-Gaddum method because it does not eliminate a gut contracting substance which is not histamine, but according to Kwiatkowski (1941) potassium. In experiments on plasma this possible error is in any case of no importance as the interfering agent is contained in the red corpuscles (Anrep et al. 1939, Kwiatkowski 1941). After extraction the plasma samples have been dissolved in distilled water and neutralized with NaOH, using bromothymol blue as an indicator. Care has been taken to obtain a pH corresponding as closely as possible to that of the Tyrode solution in the test bath.

Apart from the chemical extraction procedure an ultrafiltration method has been used. Experiments with this method have been described earlier (Emmelin 1945). The technique of ultrafiltration used is given by Rehberg (1943).

The sample is contained in a cellophane tube surrounded by a basket of metal wire, which is placed in a centrifuging tube. The filtration pressure is obtained by centrifugation. Of the different cellophane tubes recommended by Rehberg we have used the most impermeable ones. These are according to Rehberg absolutely impermeable to protein. The cellophane tubes are kept in water containing formaline. The formaline must be thoroughly removed by washing as it is known that it inactivates histamine (Kendall 1927) and makes test objects less sensitive to histamine (Best and McHenry 1930). There are two sizes of tubes, one taking about 15 ml, the other about 6 ml. Both sizes have been used but we prefer the larger which offers a greater filtration surface. As we usually have to work with small amounts of plasma we have placed a glass rod of suitable size in the middle of the tube. In this way we have, for instance, been able to fill one of the large tubes with about 5 ml of plasma using the thickest rod thus utilizing the whole filtration surface. The samples have been centrifuged at a speed of 3.000—3.500 rev. per min. for a period ranging from 30—120 minutes. Using the large tube and the thickest glass rod we obtain with the centrifuge at our disposal about 1 ml of ultrafiltrate in an hour with 5 ml of plasma. Now and then a defect in the wall of the tube may occur but this is easily discovered: a turbid, plasma coloured liquid is obtained instead of the clear colourless ultrafiltrate. In some experiments chlorazol fast pink has been used to prevent clotting and a lesion of the tube has then been still easier to detect as this dye does not pass the intact membrane.

The ultrafiltrate of plasma is slightly alkaline in comparison to the Tyrode solution used. In our earlier experiments the samples were neutralized with HCl using bromothymol blue as an indicator but this proved unnecessary as the unneutralized and the neutralized samples elicited the same contraction of the gut.

Preliminary experiments show that histamine easily passes the cellophane membrane. Testing 12 ultrafiltrates of the standard histamine solution ( $100 \gamma/1$ ) we have obtained the following values: 95, 100, 90, 90, 100, 100, 100, 100, 100, 100, 100, 100  $\gamma$  histamine per litre. Histamine is also obtained from a standard solution with added egg albumen and the yield is greater than that of the chemical extraction method (Emmelin 1945). If histamine is present in plasma it seems thus probable that it should be recovered almost quantitatively in the ultrafiltrate. The question now arises whether the ultrafiltration method also meets the second demand: to eliminate substances which could interfere with the biological assay.

Disturbing substances can be of different kinds. They might cause a contraction or a relaxation or they might change the histamine sensitivity of the gut in either direction. It should be pointed out that the typical effect of native plasma from the species investigated injected into the bath is a contraction of the gut. The corresponding ultrafiltrate causes a considerably smaller contraction (Emmelin 1945; see also figure 2 in this paper). The untreated plasma thus seems to contain a gut contracting agent apart from histamine. Its nature is unknown. It must be a compound of a large molecular size as it does not pass the cellophane membrane; the more the sample is concentrated by ultrafiltration the greater becomes the contraction released by it. This substance also differs from histamine in other respects: it causes a contraction of the gut which is not antagonized by thymoxyethyldiethylamine or theamine (figure 2). Further it makes the gut less sensitive to histamine, an effect which is reversible and disappears if the gut is washed in Tyrode solution. This effect may of course originate from some other plasma compound than the gut contracting one.

The ultrafiltration has apparently eliminated some gut stimulating agent, but there may be others which pass the membrane.

There remains the objection that the contraction elicited by the plasma ultrafiltrate might partly or wholly be due to some substance other than histamine. The experiments of chapter 3 indicate that histamine is responsible for the contraction.

Neither does there seem to be any relaxing agent of high activity present in the ultrafiltrate. If the gut is made insensitive to histamine by adding thymoxyethyldiethylamine, a plasma ultrafiltrate does not cause any relaxation (figure 2). It should be possible in this way to discover a gut relaxing substance (Emmelin 1943). The first compound to be suspected in this connexion is adenylic acid; it is, however, well-known that most of it is contained in the blood cells (Buell and Perkins 1928, Barsoum and Gaddum 1935, Billings and Maegraith 1938). It is thus not surprising that the ultrafiltration method can not be used on laked blood; the hemolysis may have liberated, apart from potassium, also adenylic acid and perhaps other interfering agents. The ultrafiltrate of plasma, however, seems to be relatively free from disturbing substances; from the preliminary report as well as from chapter 5 it is evident that ultrafiltrates and chemical extracts of plasma agree fairly well in the biological assay. It might be possible that other gut active substances are present in the ultrafiltrate of plasma but have no time to interfere with the histamine contraction, as this occurs almost instantly after the injection. It is for instance known that bile acids, which reduce the histamine sensitivity of the gut do not interfere with the estimation of histamine if the two substances are given simultaneously (Emmelin 1943).

In preliminary experiments we found that ultrafiltrates of human plasma relatively often contain an agent which interferes with the assay on gut, and human plasma has therefore not been used in this work. It is possible that the ultrafiltration method can also be used in experiments on plasma from human beings if the assay is performed on a test object which is not affected by this gut disturbing agent, e. g. perfused guinea-pig's bronchi; we are experimenting on this subject. Also in ultrafiltrates of cat's plasma an interfering substance may rarely occur as can be seen from chapter 5.

It is obvious that in experiments where some foreign sub-

stance has been added to the blood it is necessary to make sure whether this substance has been removed by the ultrafiltration and if not, whether it interferes with the assay. We have given special attention to anaesthetics and anticoagulants. In most experiments on anaesthetized animals chloralose was used. Control experiments show that chloralose in a moderate concentration does not alter the histamine sensitivity of the gut. Amongst the anticoagulants chlorazol fast pink has the advantage of not passing the ultrafiltration membrane. Heparine in concentrations generally used does not affect the gut. In earlier experiments in this laboratory it was found that extracts of heparine sometimes had a histamine-like effect on the gut. We have therefore tested each sample of heparine before using it. Sodium citrate can not be used; an ultrafiltrate of plasma from blood, which has been mixed with citrate in the usual way causes a response of the gut corresponding to at least 50 p. c. higher histamine concentration as compared with a heparinized sample. Similar results are obtained if citrate mixed with a known histamine solution is directly tested. The citrate itself causes a contraction after which the histamine sensitivity of the gut is diminished. Contrary to Minard (1941) we have found that citrate affects the histamine values even if the sample has gone through the chemical extraction procedure.

If the facts mentioned above are taken into consideration, the ultrafiltration method seems to be well suited for the preparation of plasma prior to the biological assay. To the chemical extraction method it has the advantage of being less drastic; if histamine is shown to be present in an ultrafiltrate of plasma, it can not reasonably be said that the histamine has been produced e. g. from histidine which is claimed to occur during the processes of chemical extraction (Åkerblom 1941). The ultrafiltration requires less work and supervision than the extraction processes and it also takes less time; the extraction lasts about three hours, the ultrafiltration only about one. Using a centrifuge of higher speed and improving the method in other respects we might be able to reduce the time required even more and thus perhaps get the sample ready for assay during the course of the actual experiments.

### Chapter 3

## Identification of the gut contracting substance in ultrafiltrates of plasma

»Keine der biologischen Methoden zum Nachweis von Histamin in Gewebsextrakten ist so spezifisch wie einige der Proben auf Acetylcholin» (Gaddum 1936). It seems significant that many authors avoid the term histamine and prefer to refer to »histamine-like substances», »histamine activity», »H-substance», »histamine yielding substance» etc. A disadvantage of the ultrafiltration method as compared with the extraction method is that the ultrafiltrate may contain gut contracting substances other than histamine. It thus seems imperative to ascertain to what extent the gut contracting effect of the ultrafiltrate is due to histamine. In the body there exist a number of substances which can elicit contraction of the guinea-pig gut, e. g. acetylcholine, choline, vasopressin, kallikrein, P-substance, adenosine triphosphate (Buchthal and Kahlson 1945), creatinine, tyramine, potassium, citric acid. As far as the ultrafiltrate is concerned some of these substances can be disregarded at once. Kallikrein is not ultrafiltrable and besides it is believed to occur as an inactive compound in the blood. Some of the substances are not contained in plasma, in any case not in a concentration high enough to cause contraction of the gut, e. g. acetylcholine and potassium; in our control experiments choline, creatinine and citric acid in plasma concentrations have not affected the ileum; P-substance is said not to be present in blood (Zipf and Hülsmeyer 1933). Whether there is any tyramine in blood is not definitely ascertained; Wolf and Heinsen (1935) have not been able to find it there whereas Freund (1936) believes that

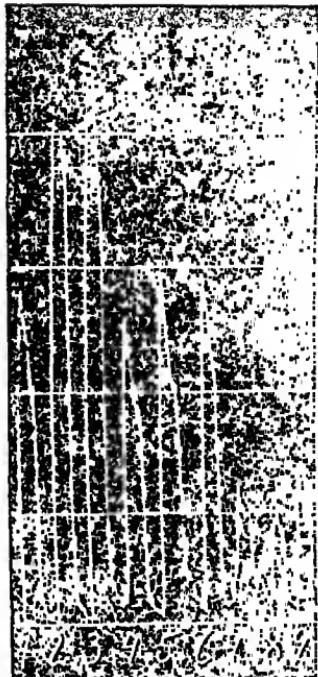


Fig. 1. Isolated guinea pig's ileum in 2 ml Tyrode solution. 1: 0.020 γ histamine. 2: 0.030 γ histamine. 3: 0.8 ml ultrafiltrate of guinea-pig's plasma. 4: 0.020 γ histamine. 5 and 6: 0.2 ml ultrafiltrate. 7 and 9: 0.1 ml ultrafiltrate. 8: 0.010 γ histamine.

the »Spätgift» of the blood is identical with tyramine. In the ultrafiltrate there may of course occur active substances which are not yet identified; Barsoum and Gaddum (1935) e. g. described an unknown gut contracting agent in blood.

In this chapter we have examined on some test objects well suited for the identification of histamine if the gut contracting agent of plasma ultrafiltrates qualitatively and quantitatively corresponds to histamine. The samples have also been tried on some test objects which are affected by some of the other gut stimulating substances mentioned above but not by histamine in concentrations which are likely to occur in plasma. Most of the experiments described in this chapter have been made with guinea-pig's plasma, but plasma from cats, dogs and rabbits has also been used.

1. *Experiments on isolated guinea-pig's ileum.* The active substance of ultrafiltrate corresponds to histamine in the following respects:

a) the contraction elicited by ultrafiltrate is exactly similar to that caused by histamine (see e. g. fig. 1 and 2). The contrac-

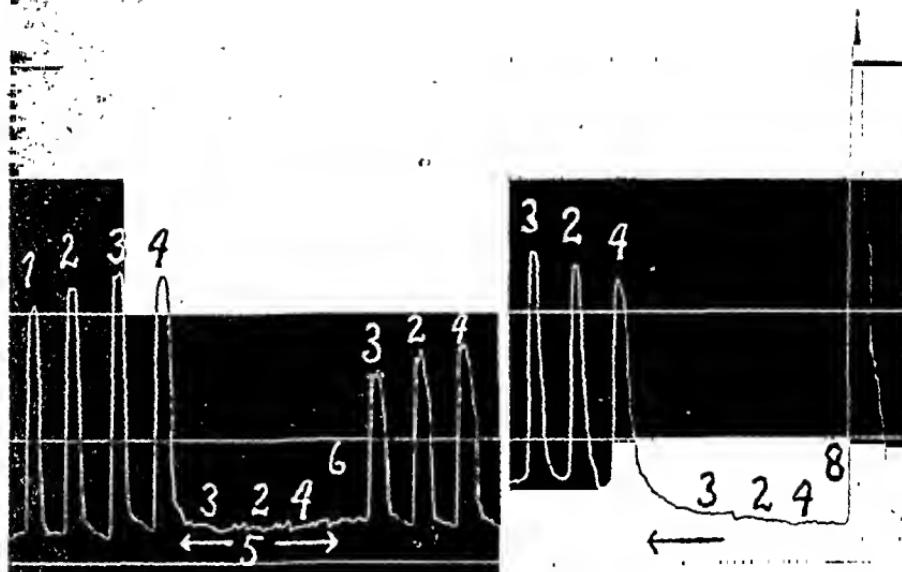


Fig. 2. Isolated guinea-pig's gut. 1: 0.020 γ histamine. 2: 0.1 ml ultrafiltrate of guinea-pig's plasma. 3: 0.022 γ histamine. 4: 0.1 ml extract of the same plasma. 5: The bath contains 0.5 γ thymoxyethyldiethylamine per ml. 6: Washing of the gut with Tyrode solution for several times. 7: The bath contains 0.5 mg theophyllinemonoethanolamine (theamine) per ml. 8: 0.1 ml untreated plasma of the same guinea-pig.

tion takes place almost instantly after the injection, soon reaching its maximum, then followed by relaxation after a single washing.

b) the contractions caused by the ultrafiltrate and by histamine follow the same concentration-action curve (fig. 1).

c) the two histamine antagonists thymoxyethyldiethylamine (Bovet and Staub 1937, Minard and Rosenthal 1939) and theophyllinemonoethanolamine (Emmelin, Kahlson and Lindström 1941) reduce the sensitivity of the gut to ultrafiltrate and histamine to the same extent (fig. 2).

d) Marthe Vogt (1943) investigating the point of attack of some drugs on rabbit's intestine found that if the gut was kept in a refrigerator for some time the nervous apparatus was destroyed prior to the muscle cells; at this state only drugs directly affecting the muscle were active. During the experiments on preserved guinea-pig's gut we tried to find out if the

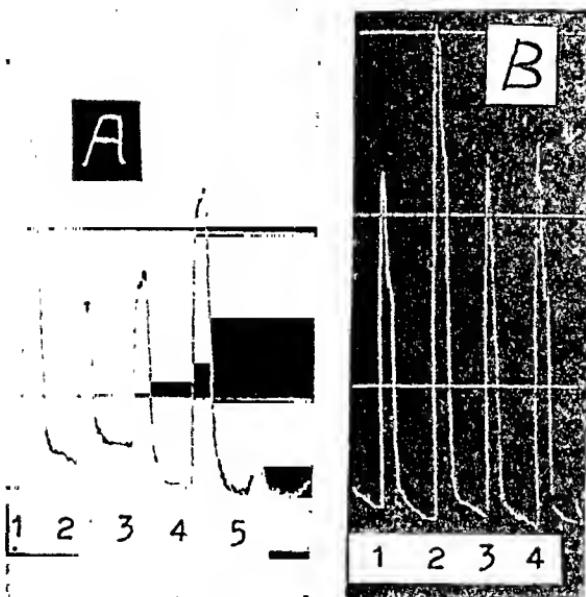


Fig. 3. Action of plasma ultrafiltrate A) on guinea-pig's gut, kept in refrigerator for 5 days and B) on the fresh gut. A. 1: 0.040 γ histamine. 2: 0.030 γ histamine. 3: 0.2 ml ultrafiltrate of guinea-pig's plasma. 4: 0.035 γ histamine. 5: 0.3 γ acetylcholine. B. 1: 0.01 γ acetylcholine. 2: 0.035 γ histamine. 3: 0.2 ml ultrafiltrate. 4: 0.030 γ histamine.

specificity to histamine can be augmented in this way as it is well-known that histamine stimulates the muscle cell directly (Gasser 1926). It has, however, been difficult to define the degree of the cold treatment when the histamine sensitivity is still high but the sensitivity to e.g. acetylcholine is lost. Yet figure 3 shows an experiment on a preserved gut, the acetylcholine sensitivity of which was greatly reduced; it is evident that the effect of ultrafiltrate on this gut is of the histamine and not of the acetylcholine type.

## 2. Experiments on blood pressure.

a) the blood pressure of the rabbit, anaesthetized with urethane, is not depressed by ultrafiltrate. It is well known that histamine in the concentrations in question does not affect the rabbit's blood pressure either.

b) like histamine ultrafiltrate depresses the blood pressure of the anaesthetized and atropinized cat. The effect of ultra-

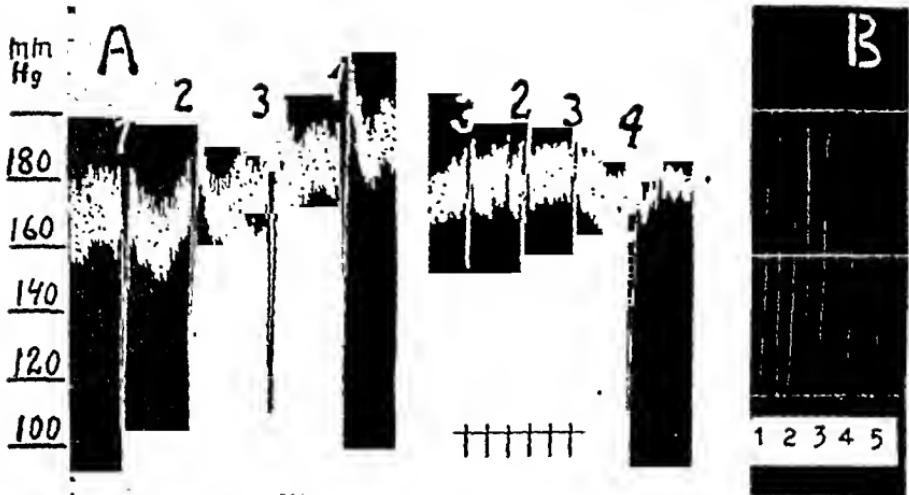


Fig. 4. Action of ultrafiltrate of guinea-pig's plasma on A) cat's blood pressure, B) guinea-pig's gut.

A. Blood pressure of cat (3.8 kg) under chloralose. The pressure in the carotid artery was registered by means of a mercury manometer. Time in minutes.

1: 0.30 γ histamine, injected into a femoral vein. 2: 1.0 ml ultrafiltrate. 3: 0.25 γ histamine. 4: 0.25 γ acetylcholine. Between the two sections of the tracing there is an interval of about 2 hours. During this time histamine was slowly injected intravenously in gradually increasing doses, beginning with 0.5 γ per kg of body weight per minute. The injection of the highest dose, 50 γ per kg per minute was interrupted 2 minutes before the second section of the tracing.

From the first part of the tracing it can be seen that the action of 1 ml of ultrafiltrate corresponds to about 0.27 γ histamine.

B. Guinea-pig's ileum. 1: 0.025 γ histamine. 2 and 4: 0.1 ml ultrafiltrate. 3: 0.020 γ histamine. 5: 0.025 γ histamine. In this assay 1 ml ultrafiltrate corresponds to about 0.23 γ histamine.

filtrate on cat's blood pressure is shown in fig. 4. It is also obvious from figure 4 that a fairly good quantitative agreement is obtained with the two test objects, cat's blood pressure and guinea-pig's ileum.

c) we have tried to work out a test specific for histamine on cat's blood pressure in the following way. In chapter 6 it will be shown that by intravenous injection of histamine in gradually increasing doses it is possible greatly to raise the histamine content of cat's plasma maintaining a normal blood pressure. This procedure diminishes the sensitivity to histamine while

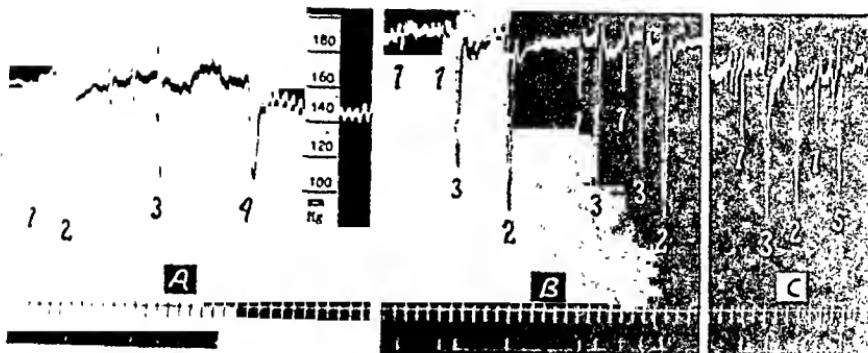


Fig. 5. Blood pressure in the femoral artery of cat (2.8 kg) under chloralose and artificial respiration. Time in minutes. 1: 0.25 γ histamine. 2: 0.80 γ acetylcholine. 3: 15 mg sodium glycocholate. At 4 a slow injection of histamine was started. At the beginning 2.7 γ histamine per kg and minute was injected, then 10 γ and 30 γ per kg and minute, each dose during 20 minutes. The injection was interrupted 3 minutes before the section B. Between B and C there is an interval of 5 minutes.

5: 2.5 γ histamine.

other depressant agents are still strongly active. We have not yet finally established the specificity of this test, but we have found that the depressant effect of two arbitrarily chosen substances, acetylcholine and glycocholic acid, is not reduced after such histamine treatment. Fig. 5 shows that slow histamine injection reduces the effect of histamine but not that of acetylcholine or the bile acid; in chapter 6 it will be seen that the sensitivity to histamine is successively restored when the histamine content of the plasma decreases to normal.

As a test of specificity Barsoum and Gaddum (1935) used the fowl's rectal coecum bathed in a strong histamine solution; the gut was contracted in the usual way by certain substances but not by histamine. The method described above seems to be of the same type as that of Barsoum and Gaddum but we hope that with our method it will also be possible to identify intravitaly liberated histamine, e.g. in reactive hyperaemia. In preliminary experiments we have found that a preparation of a certain snake venom, which exerts its depressing effect chiefly by liberating histamine from the tissues is less active on the blood pressure after slow injection of histamine.

We have made the observation that by the histamine pre-treatment the effects of ultrafiltrate and histamine are reduced to the same extent (fig. 4).

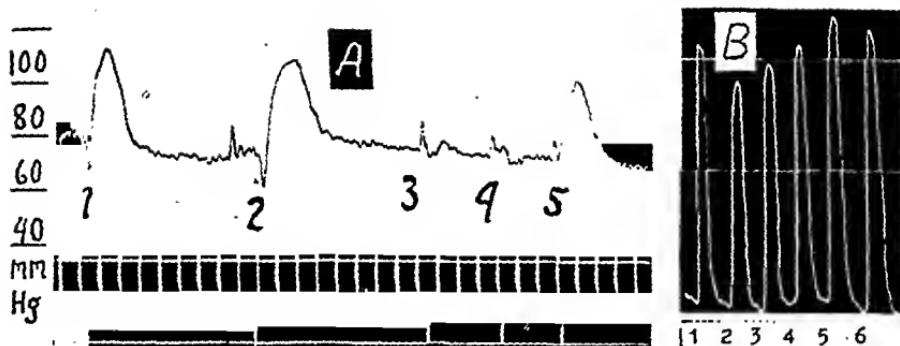


Fig. 6. Action of ultrafiltrate of guinea-pig's plasma A) on cat's blood pressure, B) on guinea-pig's gut.

A. Blood pressure in the carotid artery of a cat (2.9 kg) under chloralose and artificial respiration. Reduced circulation. Injections were made in the coeliac artery through a cannula, pointing towards the aorta. 1: 0.15 γ histamine dissolved in 0.7 ml saline solution. 2: 0.7 ml ultrafiltrate. 3 and 4: 0.7 ml saline solution. 5: 5 γ adrenaline in 0.7 ml saline solution.

In this assay the histamine content of the ultrafiltrate somewhat exceeds 200 γ of histamine per litre.

B. 1: 0.1 ml ultrafiltrate. 2: 0.015 γ histamine. 3: 0.017 γ histamine. 4: 0.1 ml ultrafiltrate. 5: 0.022 γ histamine. 6: 0.1 ml ultrafiltrate.

Result of the assay on the gut: the ultrafiltrate contains about 200 γ of histamine per litre.

d) ultrafiltrate, like histamine, under special circumstances raises the cat's blood pressure. Dale (1920) showed that histamine can stimulate the adrenaline secretion from the adrenal medulla. Feldberg (see e.g. Feldberg and O'Connor 1937) made use of this fact for the identification of histamine. Quantities as small as 0.05—0.3 γ of histamine may be detected in this way if the circulation of the cat is reduced by evisceration and histamine injected in the arterial blood stream close to the adrenals (Szczygielski 1932).

We performed such experiments as follows. In cats under chloralose the vagi were cut. V. portae, aa. mesenter. sup. et inf., a. coeliaca about one cm from aorta, were ligated. Cardia and rectum, the kidney vessels at hilus, aorta and v. cava inf. below the adrenals were also tied. A cannula was placed in the central stump of the coeliac artery, and histamine and ultrafiltrate injected.



Fig. 7. Ultrafiltrate of guinea-pig's plasma, tested on perfused guinea-pig's bronchi (A) and isolated guinea-pig's gut (B). In a guinea-pig under ether blood was withdrawn by heart-puncture. A plasma sample was ultrafiltrated. The animal was then anaesthetized with urethane subcutaneously; the bronchi were perfused and a piece of gut isolated. A. Isolated bronchi. Bronchoconstriction is indicated by larger excursions of the volume recorder. Time in minutes. 1: 0.025 γ histamine.

2: 0.3 ml ultrafiltrate. 3: 0.050 γ histamine.

According to this estimation the histamine concentration of the ultrafiltrate is something between 100 and 150 γ per litre.

B. Isolated gut. 1: 0.010 γ histamine. 2 and 4: 0.015 γ histamine. 3: 0.1 ml ultrafiltrate. 5: 0.020 γ histamine. 6: 0.017 γ histamine. Result: the ultrafiltrate contains about 160 γ of histamine per litre.

Fig. 6 shows that ultrafiltrate like histamine raises the blood pressure under these conditions. The results also show a fairly good agreement with the assay on the gut.

3. *Experiments on the isolated guinea-pig's bronchi.* Feldberg et al. (1932) found that histamine causes a bronchoconstriction in the guinea-pig's lung perfused with saline. We have tried to make use of this preparation for estimation of histamine.

The bronchial tone of a guinea-pig under urethane was registered according to Konzett and Rössler (1940). This method of registration as used in this laboratory has already been described (Emmelin, Kahlson and Wicksell 1941). In the following experiments the chest was opened and a cannula inserted through the right ventricle into the pulmonary artery. Oxygenated Tyrode solution of body temperature ran from a pressure bottle through the cannula. Injections of histamine and ultrafiltrate were made through the rubber tube close to the cannula. The perfusate ran away through another cannula in the left auricle. We have found that an injection of heparine to the animal before the pre-

paration greatly improves the method as clotting in the lung vessels is thus avoided. Unfortunately the registration of the bronchial tone is affected by an oedema of the lung developing during the perfusion, especially after injection of histamine. In an attempt to counteract this oedema we have added »dextran» (Grönwall et al. 1945) in a suitable concentration to the Tyrode solution.

The advantage of this test object is its high histamine sensitivity.

From figure 7 it is apparent that the ultrafiltrate like histamine constricts the guinea-pig's bronchi. The figures obtained are in rather good accordance with those obtained on guinea-pig's ileum.

4. *Experiments on other test objects.* Experiments have also been made on isolated rabbit's heart in Langendorff's arrangement, on frog's heart beating on a Straub's cannula, on the isolated rectus muscle and on strips of stomach of the frog. Neither ultrafiltrate nor histamine in moderate concentrations have any significant effects on these test objects.

The experiments so far referred to have shown that the biological effects of a plasma ultrafiltrate are identical with those of histamine. On testing the same ultrafiltrate on different objects a good quantitative agreement has been obtained, a fact which suggests that the biological effects are due to one and the same substance and that this substance is identical with histamine.

## Chapter 4

### Is all the blood histamine contained in the corpuscles?

It is well known that shed blood easily acquires a biological activity which is not to be found in the circulating blood. This can be induced by trauma, clotting, or simply by storing of the blood. In fresh serum there is an agent which affects blood pressure and smooth muscle; Freund (1920) called this agent »Frühgift» and Zipf (1931) identified it as adenylic acid. It is rapidly destroyed enzymatically, and the serum then causes other effects, due to »Spätgifte» ( $\beta$ -thrombovasin according to Simon 1938, Simon and Komlos 1939); Freund (1936) suggests that these »Spätgift»-effects are caused by tyramine which, however, is denied by Simon (1937). These unidentified substances are believed by the authors quoted above, normally to be present in the platelets; they are liberated when the platelets are destroyed by shaking, by clotting or by mere storing of the blood. Gut contracting agents have recently been found in serum and platelets by Zucker (1944) and Tsai, McBride and Zucker (1944). Many investigators have observed that blood, injured by shaking, hemolysis or standing can cause circulatory effects (Phemister and Handy 1927, Feldberg, Flatow and Schilf 1929, Newton 1932, Fleisch 1937). Shaking or clotting augments the adenosine activity of blood (Barsoum and Gaddum 1935). The substances discussed here may, of course, interfere with our tests.

Histamine is of special interest in this connexion. O'Connor (1912) found that serum has certain effects on smooth muscles which plasma has not. He suggested that the active agent

originated from the platelets and also stressed that its effects were very similar to those of the recently discovered  $\beta$ -imidazolylethylamine. Schwartz (1936) found that after clotting of the rabbit's blood histamine appears in the serum and he believed that the histamine originated from the platelets; he also suggested that shaking liberates histamine. Barsoum and Smirk (1936) showed that the histamine content of plasma increased if the blood was kept for some time before removing the corpuscles by centrifugation. According to Code (1937) most of the blood histamine can be recovered from serum. Code also observed that if clotting is prevented by potassium oxalate and the blood is left standing for some time the histamine concentration in plasma is increased.

Taking all these observations into account, and also the fact that most of the blood histamine, at least in the rabbit, is contained in the extremely fragile platelets it is tempting to assume that all the histamine, which is found in a plasma sample, originates from the platelets of the circulating blood. It must be born in mind that trauma and clotting, which are known to liberate active substances, are at work during the procedures involved in the securing of a sample of blood plasma. O'Connor (1912) pointed out the difficulties of avoiding injuries to blood cells at the blood collection. Minard (1941), who found that of the total blood histamine in the rabbit only 2—3 per cent was present in the plasma, suggested that the plasma histamine might have been liberated from platelets destroyed outside the body. Barsoum and Smirk (1936) also discussed the possibility that histamine detected in a plasma sample is liberated from the blood cells *in vitro*.

In a series of experiments we have tried to find out to what extent a rough handling of the blood affects the plasma histamine values. We have also investigated the effect of clotting, since Anrep et al. (1939), contrary to Code, claim that on clotting, histamine does not appear in the serum but can be extracted from the fibrin fraction. In a second series of experiments we have tried to establish whether or not histamine is a normal constituent of plasma.

1. *The effect of hemolysis.* In a preliminary experiment we

obtained a plasma sample rich in platelets in the following way. From an ear vein of a rabbit blood was collected in a glass tube containing heparine and was then slowly centrifuged for a short time (1000 rev./min. during 15 min.). A sample of the turbid plasma was withdrawn and the blood then centrifuged at a higher speed until the plasma became clear (3500 rev./min. during 30 min.). A five-fold volume of distilled water was added to the turbid plasma and the sample was ultrafiltrated. The clear plasma was also ultrafiltrated. The assay on the guinea-pig's gut showed that the turbid plasma contained about 2000  $\gamma$  of histamine per litre, the clear plasma 185  $\gamma$ /l. It is obvious that the lysis of the corpuscles is sufficient to liberate histamine; since the ultrafiltrate contained physiologically active histamine, this histamine must have been present in the corpuscles in an active state or as a very labile inactive compound.

2. *The effect of shaking.* A sample of heparinized rabbit's blood was divided in two portions. One was centrifuged immediately, the other after having been moderately shaken a few times in the tube. The experiments were carried out with blood from three rabbits. The results of these experiments are given in table 1, where plasma A is obtained from the unshaken, plasma B from the shaken sample. The plasma samples were extracted (experiment nr 1 and 2) or ultrafiltrated (experiment nr 3) and the histamine content was determined. It is obvious that in rabbits the plasma histamine concentration increases on shaking.

TABLE I.

Experiment nr	Hi content, $\gamma$ /l		
	plasma A	plasma B	blood
1	840	1500	2500
2	200	2200	1950
3	150	600	-

3. *The effect of clotting.* From the ear vein of rabbits, blood was collected alternately in two centrifuging tubes, one of which

contained heparine. The samples were left standing for a few hours at room-temperature. The heparinized blood remained liquid whereas the other soon clotted and serum and clot began to separate. The samples were centrifuged; the clear fluids were collected and the histamine content estimated after ultrafiltration. Table 2 indicates that in our experiments the histamine content of rabbit's serum is considerably larger than that of plasma.

TABLE 2.

Experiment nr	1	2	3	4	5	6
Hi content plasma	800	630	250	560	500	150
γ/l serum	4000	3000	1100	1100	3000	2500

There is every reason to assume that at least most of the gut activity of serum is due to histamine: the effect of serum ultrafiltrate is antagonized by thymoxyethyldiethylamine; the ultrafiltrate also depresses the blood pressure of the atropinized cat to an extent which in terms of histamine corresponds to the activity on the gut. Besides, histamine has been chemically identified in the »white layer» of blood from the rabbit (Code and Ing 1937).

4. *The histamine content of plasma from the same individual over a period.* It is well known that the histamine concentration of blood, while varying within fairly wide limits in a species, remains rather constant during a long period in one and the same individual (Code and Mac Donald 1937, Zon, Ceder and Crigler 1939, Rose and Browne 1940, Minard 1941). If the histamine detected in a plasma sample in vitro originates from the corpuscles it seems likely that subsequent plasma samples from one individual should — contrary to the blood samples — show great variations in histamine content owing to greater or smaller injury to the blood on different occasions. During a period we have estimated the histamine concentration of plasma and of blood in a number of animals. Hypothesing that histamine occurs in the circulating plasma nothing is known

as to whether it occurs there in constant amounts as is the case in total blood. We have investigated to what extent the histamine concentration of other tissues than blood is maintained constant; excised pieces of skin have been examined. The experiments were carried out on guinea-pigs, rats, dogs and rabbits.

a. *Experiments on guinea-pigs.* The figures from 9 animals are summarized in table 3. The body weight of each animal at the beginning of the experiment is also given. The blood was collected from the etherized animal by heart puncture; 5—9 ml of blood was withdrawn in a syringe, containing heparine. 1—2 ml of blood was extracted. The remaining blood was centrifuged at 3500 rev. per min. for 15—30 minutes and plasma was collected and extracted. An area of the abdominal skin was carefully shaven and a piece of skin excised, after which the wound was closed with sutures. The subcutaneous tissue was carefully removed from the excised skin which was then weighed as soon as possible to prevent loss of weight due to evaporation of water (the skin samples usually weighed about 100 mg). The skin was then minced with scissors and ground in a mortar with quartz powder and trichloroacetic acid for 30 minutes. After centrifugation the sample was extracted according to Code. Samples of plasma, blood and skin were assayed on guinea-pig's ileum.

It is obvious from the table that the histamine concentration not only of blood and skin but also of plasma is kept at a fairly constant level in an animal for weeks and months. Considering that in these experiments the plasma histamine is sustained at a rather constant level in one and the same individual, it is not unlikely that the circulating plasma really contains histamine; and further, it is tempting to assume that some regulating mechanism endeavours to keep the histamine concentration of plasma, like that of total blood and skin, at a constant level. This explanation seems to us more reasonable than e.g. the assumption that the blood collection should always cause the same degree of damage and that the cells should possess a fragility, typical to each individual but varying within wide ranges in a species.

TABLE 3.

Guinea-pig nr and body-weight	Date of experiment	Histamine content of		
		plasma, γ/l	Blood, γ/l	Skin, mg/kg
1 240	26.2	200	615	—
	7.3	220	600	11.4
	10.4	180	580	13.9
	26.4	225	600	10.8
	8.5	200	570	—
2 635	1.3	200	700	6.5
	14.3	225	—	5.7
	27.3	—	750	7.4
	6.4	210	700	—
3 780	1.3	135	430	2.5
	13.3	150	400	1.9
	9.4	170	410	2.3
4 840	3.3	325	810	—
	27.3	335	900	4.5
	10.4	290	840	5.8
	24.4	—	800	6.2
5 870	5.3	125	450	—
	27.3	125	500	4.8
	11.4	—	400	3.8
	30.5	135	520	—
	26.7	180	—	—
6 690	6.3	250	475	11.4
	26.3	265	450	13.9
	17.4	—	450	—
7 690	14.3	—	375	3.3
	28.3	150	340	2.8
	12.4	130	325	3.1
	27.4	150	360	—
8 460	15.3	320	670	12.5
	10.4	300	650	14.9
9 425	16.3	225	510	—
	28.3	—	550	11.3
	16.4	250	450	14.7

b. *Experiments on rats.* 7 big white rats were used for these experiments which were carried out similar to those on guinea-pigs. 3—6 ml of blood was obtained by heart puncture; the

blood was used for estimation of histamine in plasma. The skin samples weighed 25—75 mg. The results, summarized in table 4, agree with those of table 3.

TABLE 4.

Rat nr	1			2			3		
Date	19.4	3.5	30.5	23.4	2.5	17.5	19.4	2.5	24.5
Plasma Hi, $\gamma/l$	140	145	150	250	225	225	150	165	150
Skin Hi, $\gamma/l$	24.8	27.9	22.3	30.0	32.0	35.5	19.5	16.5	20.5
Rat nr	4			5			6		
Date	18.4	17.5	20.4	2.5	19.4	17.5	17.4	3.5	1.6
Plasma Hi, $\gamma/l$	110	100	190	220	315	300	145	145	165
Skin Hi, $\gamma/l$	10.9	15.0	—	33.7	37.5	32.8	28.2	31.9	27.0

c. *Experiments on dogs.* The histamine content of plasma was determined in two dogs. In an ear vein of a nonanaesthetized dog an incision was made from which about 20 ml of blood was collected in a centrifuging tube, containing heparine. The sample was treated as in the previous experiments. Table 5 demonstrates the constancy of the plasma values.

TABLE 5.

Dog nr	1				2			
Date	20.2	2.3	15.3	7.4	27.2	6.3	8.3	18.5
Plasma Hi, $\gamma/l$	40	35	40	40	60	60	55	55

d. *Experiments on rabbits.* 10—15 ml of blood was collected from an ear vein of the nonanaesthetized animal. From table 6 it is obvious that the histamine concentrations of the plasma samples show very great variations while the histamine content of blood remains fairly constant.

In this respect the rabbit obviously differs from the other

TABLE 6.

Rabbit nr	1						2			
Date	9.2	19.2	13.3	18.4	25.5	30.5	10.3	26.3	12.4	23.4
Plasma Hi, $\gamma/l$	570	800	800	600	200	380	390	1900	140	280
Blood Hi, $\gamma/l$	2500	3000	—	—	—	2800	3800	—	—	4000
Rabbit nr	3			4			5			6
Date	18.1	23.1	24.1	29.4	3.5	3.6	9.6	20.3	29.5	11.7
Plasma Hi, $\gamma/l$	170	800	150	1200	300	200	150	750	210	250
Blood Hi, $\gamma/l$	4200	4500	3300	2800	—	—	—	3800	4200	4200

species investigated. It may be pointed out that in a paper by Chute and Waters (1941), not directly concerned with the subject under discussion, we have found very great variations of the plasma histamine in one and the same rabbit. In rabbits the platelets have an exceptionally high histamine content and liberate histamine very easily *in vitro*. It is reasonable to assume that the histamine, which can be extracted from the rabbit's plasma, mainly originates from the platelets. We have investigated the plasma histamine in experiments where the blood was handled as carefully as possible. It may be mentioned that the plasma values in table 6 are of the same order as those given by other authors (e. g. Zon, Ceder and Crigler 1939, Chute and Waters 1941, Rocha a Silva and Andrade 1943). Table 7 summarizes 6 experiments of this kind. The first plasma sample (1) was obtained in the usual way, the second (sample 2) as follows. The blood was collected from ear veins of nonanaesthetized animals (rabbits nr 7, 8 and 9) or from the carotid artery of animals under urethane (rabbits nr 10, 11 and 12). The blood was collected in a paraffined centrifuging tube containing heparine and cooled in ice water. The sample was centrifuged. In some cases the tube was placed in a larger centrifuging tube, containing ice water in order to cool the sample during the centrifugation. The tube was paraffined as this is known to protect the platelets. The blood was cooled

because heating may release histamine from cells and cellular debris (Trethewie 1938).

TABLE 7.

Rabbit nr		7	8	9	10	11	12
Hi content γ/l	sample I	380	340	450	840	350	750
	sample II	135	75	180	65	80	210

The table shows that plasma obtained under these conditions has a relatively low histamine content. When using a similar method, Minard (1941) also found a low histamine concentration in rabbit's plasma. In experiments with blood from the rabbit it is obviously of great importance to protect the corpuscles from injuries. In experiments with other species the plasma histamine does not decrease when special precautions are taken to protect the corpuscles. Only in rabbits we have investigated blood from one and the same animal, treated in the two different ways; it may, however, be mentioned that the average plasma histamine content of 14 guinea-pigs, the blood of which had been obtained in the usual way, was 191 γ per litre, while the average of 23 animals, the blood of which had been treated according to the more careful method, was 196 γ per litre.

These experiments show that the plasma histamine in guinea-pigs, rats and dogs, as determined by these methods, is kept fairly constant in the same individual and they indicate that histamine is a normal constituent of plasma. This is supported by the fact that histamine is present in aqueous humour (Emmelin and Palm 1944; further experiments are described in chapter 5). Even if the conception that the aqueous humour is produced from plasma by an ultrafiltration process is not accepted by all investigators, there is no reason to suppose that the histamine present in the aqueous is not derived from the plasma. A similar assumption was made by Barsoum and Smirk (1936), who detected histamine in oedema fluid from patients with congestive heart failure; from this fact they concluded that the

histamine found by them in plasma of these patients had not leaked out of the corpuscles *in vitro*.

Contrary to the other species investigated the rabbit shows great variations in the plasma histamine concentration of one and the same individual. In this animal the plasma histamine can be greatly decreased by careful handling of the blood. The question arises whether rabbit's plasma normally contains any histamine at all. Some facts indicate that this is the case. In rabbits the aqueous humour contains histamine. By careful handling of the blood, histamine values are obtained which are almost as low as those of aqueous humour. In chapter 5 it will be shown that by using a special technique — ultra-filtration *in vivo* — histamine values of the rabbit's plasma are found which are just as low as those of aqueous humour — but not lower.

## Chapter 5

### Is the plasma histamine present in a physiologically active or inactive form?

Several authors have stressed that using the common methods of extraction it is not possible to decide whether histamine detected with the biological test objects was really present in the circulating blood in a free, active form (Gaddum 1936, Barsoum and Smirk 1936, Feldberg 1937, Dale 1937—38, Code and Mac Donald 1937). It should be remembered that the biological assay is proceeded by drastic extraction processes during which the sample i. a. is boiled for  $1\frac{1}{2}$  hours in strong hydrochloric acid. It has even been suggested that the histamine found is derived from histidine, which has been decarboxylated during the extraction (Akerblom 1941). Dale (1936) suggests that the plasma histamine may, like kallikrein, form an inactive compound with some plasma component and Holtz (1937) presents a similar hypothesis. Kaiser (1939) is of the opinion that the plasma histamine is physiologically inactive and probably adsorbed to protein.

It is *a priori* not improbable that histamine is present in plasma as a physiologically inactive compound. It is known that histamine can occur in a conjugated form in the body. In the urine Anrep et al. (1944) found a compound from which histamine could be released by acid hydrolysis. It is not yet clear in which state histamine is present in the cells but many investigators suggest that it exists as some inactive compound. Rocha e Silva (1943) is of the opinion that the histamine of the cells is bound to protein in a peptide linkage, the proximal amino acid in the chain being either arginine or lysine.

As plasma can not, without previous treatment, be tested on the usual test objects we have approached the problem under discussion by using a preparation method which is not so drastic as the chemical method; we have applied the ultrafiltration method. Even in this process histamine might be liberated from a hypothetic, very labile compound, and we have therefore also tackled the problem in other ways.

### 1. *Ultrafiltration of plasma samples.*

In these experiments a plasma sample was divided into two portions, one of which was ultrafiltrated, the other extracted according to Code. The extracts were dissolved in distilled water to the same volume as that of the original plasma sample. The histamine values are given in terms of  $\gamma$  per litre ultrafiltrate or extract. The assay was made on guinea-pig's gut. Some samples have for the purpose of identification been treated according to the methods of chapter 3. Some of the results given here have already been published in a preliminary report (1945).

a. *Experiments on guinea-pigs.* The blood was obtained from the etherized animal by heart puncture as described in chapter 4. As the plasma sample had to be large enough for both extraction and ultrafiltration a somewhat greater amount of blood (10—15 ml) was usually withdrawn. The results of 38 experiments are shown in table 8.

b. *Experiments on rats.* Blood was collected as in chapter 4. The results of 12 experiments are given in table 9.

c. *Experiments on cats.* On cats under chloralose or ether 15—30 ml of blood was withdrawn through a cannula in the femoral artery. The blood was collected in a cooled, paraffined centrifuging tube containing heparine. Since it has been stated that the histamine content of blood is increased at the beginning of a chloralose narcosis (Kwiatkowski 1943) the blood samples were taken  $1\frac{1}{2}$ —2 hours after the injection of chloralose. Table 10 shows the results of experiments on 14 cats.

d. *Experiments on dogs.* The blood was obtained from the ear vein of unanaesthetized dogs (nr 1 and 2 of table 11) or

TABLE 8.

Guinea-pig nr		1	2	3	4	5	6	7	8	9	10	11	12	13
Histamine content, γ/l	U. f.	180	250	260	140	150	150	185	240	330	165	310	140	325
	extr.	190	295	240	—	160	140	170	250	320	155	340	135	335
Guinea-pig nr		14	15	16	17	18	19	20	21	22	23	24	25	26
Histamine content, γ/l	U. f.	275	250	130	140	130	150	160	190	225	220	150	125	115
	extr.	260	—	115	150	150	135	165	175	235	210	170	115	110
Guinea-pig nr		27	28	29	30	31	32	33	34	35	36	37	38	—
Histamine content, γ/l	U. f.	130	90	200	115	250	350	80	190	350	160	200	200	—
	extr.	130	100	200	100	230	375	80	210	315	160	200	175	—

TABLE 9.

Rat nr		1	2	3	4	5	6	7	8	9	10	11	12
Histamine content, γ/l	U. f.	150	150	110	330	160	215	175	240	155	155	110	160
	extr.	150	145	110	315	140	190	155	225	145	145	110	165

TABLE 10.

Cat nr		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Histamine content, γ/l	U. f.	50	30	35	80	35	25	40	75	45	55	150	60	45	50
	extr.	50	25	35	65	30	25	30	40	30	40	25	50	35	50

TABLE 11.

Dog nr		1	2	3	4	5	6
Histamine content, γ/l	U. f.	35	55	60	55	35	60
	extr.	40	55	60	50	30	65

from the femoral artery of animals under morphine-chloralose. The results are summarized in table 11.

e. *Experiments on rabbits.* The blood was obtained from the ear vein of unanaesthetized rabbits or from the carotid artery of rabbits under urethane or chloralose. Table 12 shows 28 experiments on rabbits.

TABLE 12.

Rabbit nr		1	2	3	4	5	6	7	8	9	10
Histamine content, $\gamma/l$	U. f.	620	145	2000	1900	810	800	560	1400	310	175
	extr.	600	145	2000	1900	800	800	570	1200	350	150
Rabbit nr		11	12	13	14	15	16	17	18	19	20
Histamine content, $\gamma/l$	U. f.	800	170	150	200	280	185	800	300	420	330
	extr.	800	—	150	200	—	200	840	310	390	330
Rabbit nr		21	22	23	24	25	26	27	28	—	—
Histamine content, $\gamma/l$	U. f.	360	130	75	85	200	250	75	150	—	—
	extr.	380	130	65	80	210	250	75	135	—	—

In experiments nr 21—28 of table 12 the same precautions were taken as in chapter 4 at the blood collection.

It is obvious from the tables that the ultrafiltration and the extraction methods give approximately similar figures. (In the case of cat there are some exceptions, the reason for which is obscure; the ultrafiltrate in these instances may contain some agent interfering with the assay.) We have not gone into a detailed study of the concordance of the two methods of preparing plasma samples and the discrepancies observed, since this does not touch our primary problem. Our experiments are designed to show that plasma contains histamine in an ultrafiltrable form; the amount of ultrafiltrable histamine corresponds fairly well to that which can be obtained by the chemical extraction method. This fact obviously excludes the possibility that the histamine is produced e. g. by decarboxyla-

tion of histidine at the chemical treatment of the plasma. On the other hand these experiments do not definitely prove that histamine is present in the circulating plasma in a free, active state. There remains the possibility that the histamine occurs physiologically in an adsorbed form or as an extremely labile compound and is liberated during blood collection, centrifugation of the blood or ultrafiltration. It is, however, unlikely that histamine is gradually liberated during ultrafiltration since the histamine concentration of the filtrate is the same at the beginning as at the end of the filtration. In a series of experiments to be described later we have obtained plasma samples for biological assay avoiding by special measures the trauma of blood collection and centrifugation.

## 2. *The histamine content of aqueous humour.*

The mode of production of aqueous humour still remains controversial in essential points (for references see e.g. Hodgson 1938, Davson 1939, Robertson and Williams 1939, Davson and Quilliam 1940, Duke-Elder and Davson 1948, Friedenwald 1944). Many investigators consider the aqueous to be an ultrafiltrate of plasma. It must be of special interest to study the histamine concentration of this filtrate, produced within the organism, where the blood is not subjected to the treatment which is necessary for the preparation of an ultrafiltrate *in vitro*. It has already been shown that the aqueous can be tested directly on guinea-pig's gut without any previous treatment (Emmelin and Palm 1944). The aqueous humour is usually slightly alkaline as compared to the Tyrode solution used, but we have observed that neutralization does not alter the histamine values found, and we have therefore omitted this procedure. The aqueous was obtained by puncturing the anterior chamber of the eye with a fine needle. In many cases the aqueous was injected into the test bath immediately after the aspiration, and its histamine concentration determined. When a plasma sample of the same animal was tested for comparison the aqueous was kept in a refrigerator for a few hours. Usually the aqueous from both eyes were mixed.

a. *Experiments on guinea-pigs.* Aqueous humour and blood were obtained from etherized guinea-pigs. The total amount of aqueous from one animal was only about 0.1 ml and it must be emphasized that the histamine values obtained must be considered as very approximate since the available quantity was only sufficient for one test on the gut. Table 13 shows the results of 24 experiments on guinea-pigs.

TABLE 13.

Guinea-pig nr		1	2	3	4	5	6	7	8	9	10	11	12
Histamine content, γ/l	aqueous	200	250	175	175	200	150	125	300	300	190	150	110
	plasma U. f.	190	250	175	160	—	—	165	330	240	—	—	—
	plasma extr.	175	230	—	165	—	—	155	320	250	—	150	—
Guinea-pig nr		13	14	15	16	17	18	19	20	21	22	23	24
Histamine content, γ/l	aqueous	125	200	140	160	250	175	250	300	95	200	225	250
	plasma U. f.	185	175	130	140	220	—	—	250	—	175	200	225
	plasma extr.	170	—	—	150	—	—	—	—	—	—	—	175

b. *Experiments on rats.* Also on rats the samples were collected from the etherized animals. From both eyes only a total of 0.03—0.05 ml of aqueous could be obtained. It is obvious that the assay with such small quantities yields only very approximate values. Besides, a specially histamine sensitive piece of gut was required and we have therefore only been able to assay a few of the samples taken. Table 14 summarizes the results of 6 experiments.

TABLE 14.

Rat nr		1	2	3	4	5	6
Histamine content, γ/l	aqueous	350	200	130	275	100	200
	plasma U. f.	—	175	110	330	150	150
	plasma extr.	310	155	—	310	145	150

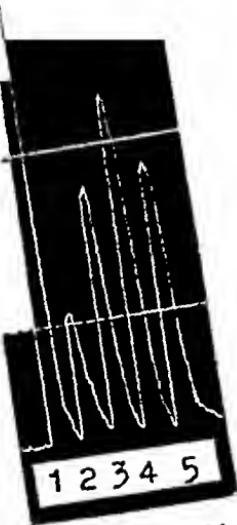


Fig. 8. Isolated guinea-pig's gut. 1: 0.01 γ histamine. 2: 0.002 γ histamine.  
3: 0.03 ml of rat's aqueous humour. 4: 0.005 γ histamine. 5: 0.004 γ histamine.

Fig. 8 illustrates the estimation on a piece of gut extremely sensitive to histamine (experiment nr 3 of table 14).

c. Experiments on cats. From cats under chloralose about 0.5 ml of aqueous was obtained from each eye. The results of experiments on 22 cats are given in table 15.

d. Experiments on dogs. The aqueous of 4 dogs was investigated. From one eye of a dog about 0.8 ml of aqueous could be obtained. Table 16 gives the results of these experiments.

e. Experiments on rabbits. We investigated 7 rabbits under urethane or chloralose. Each eye yielded about 0.3 ml of aqueous. In one of the rabbits the aqueous contained 35 γ of histamine per litre, the ultrafiltrated 185 and the extracted plasma 200 γ per litre. In another rabbit the corresponding figures were: 65, 200 and 210 γ per litre. Figures from the remaining 5 rabbits are found in table 17. All the blood samples were collected with the precautions described in chapter 4.

TABLE 15.

Cat nr		1	2	3	4	5	6	7	8	9	10	11
Histamine content, $\gamma/l$	aqueous	40	60	60	50	25	35	65	45	45	50	35
	plasma U. f.	45	75	—	—	—	25	—	35	45	—	35
	plasma extr.	30	40	—	—	—	25	60	—	—	40	35
Cat nr		12	13	14	15	16	17	18	19	20	21	22
Histamine content, $\gamma/l$	aqueous	50	55	45	45	50	45	25	60	150	50	45
	plasma U. f.	—	—	35	45	60	55	60	60	150	60	50
	plasma extr.	50	45	—	—	—	—	—	50	25	40	50

As has already been pointed out the figures given here for the histamine content of aqueous are rather approximate, especially in rats and guinea-pigs. The results, however, indicate that the histamine concentration of aqueous and plasma is of the same order. In rabbits the histamine concentration of aqueous is considerably smaller than that of plasma. This can be explained on the assumption that in spite of very careful handling of the blood a certain break-down of platelets occurs. If this is true the histamine content of the aqueous should give useful information about the histamine concentration in the circulating plasma.

The fact that biologically active histamine can be demonstrated in native aqueous humour — a fluid produced from plasma within the body by a process thought to be an ultrafiltration — and that the aqueous and plasma contain histamine in approximately the same concentration, support the view that histamine is present in plasma in an active state. It should be remembered, however, that other processes than ultrafiltration might be involved in the production of the aqueous. There are some indications that secretory processes are active. In the following experiments we have made the body itself produce an ultrafiltrate of plasma using a membrane of defined permeability.

### 3. Ultrafiltration *in vivo*.

In these experiments we used the ordinary arrangement for ultrafiltration *in vitro*. A stopper was placed in the opening of the cellophane tube and through it two thin glass tubes were inserted, a short and a long one reaching almost to the bottom of the cellophane tube. The glass tubes were connected with cannulae by means of short rubber tubings. The first experiments were carried out on dogs under morphine-chloralose. In these experiments heparine was injected intravenously to prevent clotting. The femoral artery was exposed in two places on the thigh. In the central part of the artery one of the cannulae of the ultrafiltration system was inserted and blood was allowed to fill the system. A clip was then put on the artery centrally to the cannula and left there for the short time during which the other cannula was inserted peripherally in the artery. When the preparation was ready the clip was removed and the blood flowed through the leg passing the filtration system. Ultrafiltration was thus carried out, the blood pressure of the animal acting as filtration pressure. A clear, colour-less fluid accumulated in the centrifuging tube. To prevent evaporation the opening of the tube was closed with paraffin.

TABLE 16.

Dog nr	Ultrafiltration <i>in vivo</i>			Histamine content, γ/l		
	time of collection hours	volume ml	histamine content γ/l	aqueous	plasma U. f.	plasma extr.
1	3½	0.2	60	65	60	60
2	3	0.4	55	45	55	50
3	3	0.5	35	35	35	30
4	4	1.3	60	60	60	65

Experiments on 4 dogs are summarized in table 16. The histamine concentration was determined in *in-vivo-ultrafiltrate*,

aqueous humour and in ultrafiltrate and extract of plasma from shed blood.

It seemed of special interest to experiment on rabbits with this technique because of the discrepancy between the histamine values of aqueous humour and ultrafiltrate of plasma. In these experiments the ultrafiltration system was connected with the carotid artery of animals under urethane or chloralose. Table 17 shows 5 experiments on rabbit. Fig. 9 illustrates some details of the assay in experiment nr 2 of table 17.

TABLE 17.

Rabbit nr	Ultrafiltration in vivo			Histamine content, γ/l		
	time of collection hours	volume ml	histamine content γ/l	aqueous	plasma U. f.	plasma extr.
1	4	0.2	50	50	85	80
2	4	0.4	60	55	150	135
3	3	0.4	40	40	75	75
4	3	0.9	55	50	135	120
5	4	1.1	70	70	290	280

From the two tables it is evident that the volume of ultrafiltrate obtained varied considerably. In the first experiment on dog and rabbit a small cellophane tube was used, in the following experiments a larger one. As the blood pressure was registered only in a few of these experiments, we can give no information of the part played by this factor.

In these experiments an ultrafiltrate of blood plasma was secured with a minimum of damage to the blood. It is of interest to note that in rabbits the ultrafiltration in vivo yields a fluid, the histamine concentration of which is smaller than that of the ordinary plasma ultrafiltrate and equal to that of the aqueous humour.

The presence of biologically active histamine in in-vivo-ultrafiltrates in a concentration equal to that of aqueous and

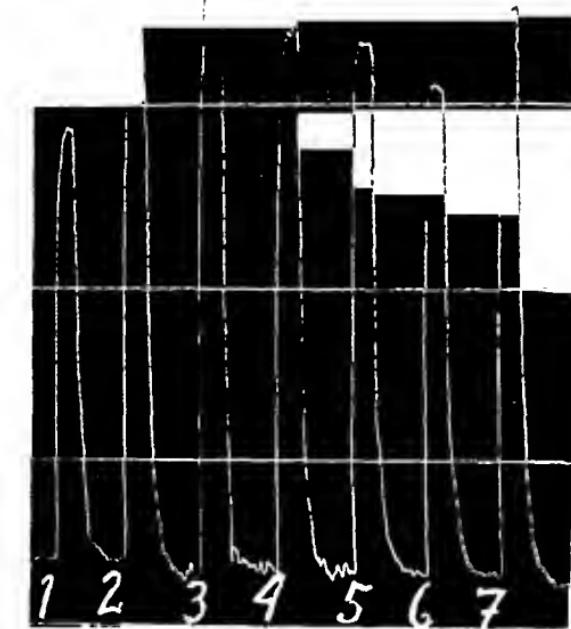


Fig. 9. Isolated guinea-pig's gut. 1: 0.010  $\gamma$  histamine. 2 and 4: 0.2 ml of in-vivo-ultrafiltrate from rabbit's blood. 3: 0.014  $\gamma$  histamine. 5: 0.012  $\gamma$  histamine. 6: 0.2 ml of aqueous from the same rabbit. 7: 0.012  $\gamma$  histamine.

in dog also to that of plasma gives additional support to the conception that histamine is present in plasma in an active state, or if not, as a compound which extremely easily liberates active histamine.

#### *4. Cross circulation experiments.*

Still another mode of attacking our problem can be founded on the fact, demonstrated in chapter 4, that the histamine concentration of plasma is kept at a fairly constant level in one and the same individual but varies greatly within the species. If histamine is present in plasma in an active form it should be possible to elicit typical histamine effects in an animal with a low plasma histamine concentration by transfusion of blood from an animal with high histamine concentration. On this assumption we have performed cross circulation experiments in

guinea-pigs. These animals are for several reasons well suited for experiments of this kind. It is fairly easy to determine the plasma histamine content in a great number of these animals. Further, there is a tissue to be found in guinea-pigs highly suitable as a test object for histamine, the bronchi, which is both sensitive and well adapted to this special type of experiment because it gives — contrary e. g. to the cat's blood pressure — a marked reaction to a rather slowly occurring increase of the blood histamine concentration. This difference between the bronchi and the blood pressure as test objects for histamine has been pointed out by Emmelin, Kahlon and Wicksell (1941).

The experiments were carried out in the following way. Two selected guinea-pigs were anaesthetized with urethane subcutaneously. The bronchial tone of both animals was registered according to Konzett and Rössler (1940). Heparine was injected intravenously. In the first experiments the cross circulation was arranged as follows. Two cannulae were connected by a short piece of rubber tubing obstructed by a clip. This system was filled with Tyrode solution containing heparine. One cannula was inserted in the central stump of the carotid artery of one animal, the other in the jugular vein of the other, the cannula pointing towards the heart — and vice versa. When the clips were simultaneously removed a cross circulation was thus established, blood flowing from the carotid artery to the jugular vein of the other animal. A disadvantage of this method is that a large arteriovenous anastomosis is formed and the capillary net thus deprived of an amount of arterial blood. Considering the short duration of the experiment this disadvantage might be of no consequence, but in later experiments another technique was used for safety. On each animal the longest possible section of the carotid artery was freed and two cannulae, connected by a piece of rubber tubing inserted, one pointing centrally, the other peripherally thus allowing blood to flow through the system to the periphery. Each of these cannulae had a side tube. By means of a short piece of rubber tubing the central cannula of one animal was connected with the peripheral cannula of the other. The whole

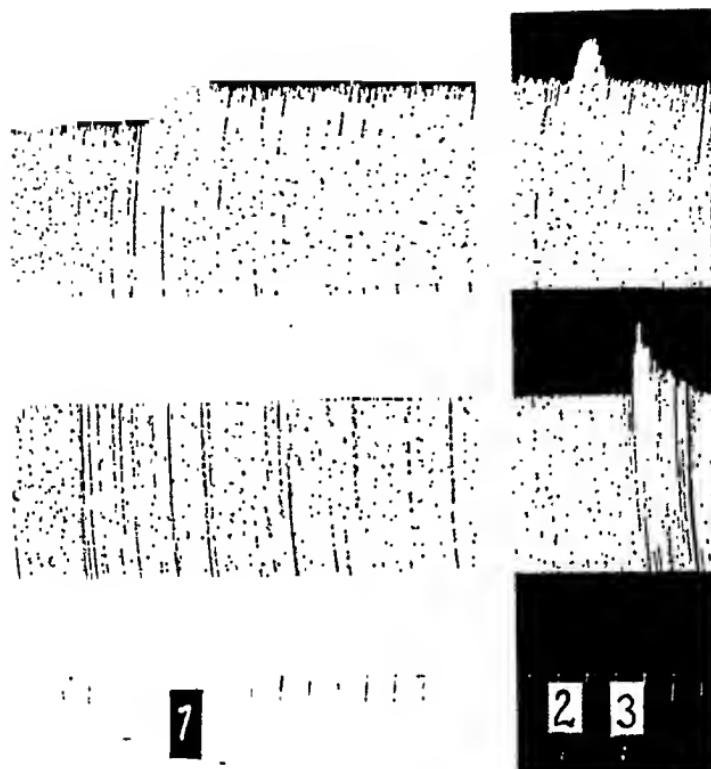


Fig. 10. Cross circulation experiment (second method). From above: bronchial tone of guinea-pig A and of guinea-pig B, time in minutes and signal marks. Between the two sections of the tracing there is an interval of 8 minutes. 1: cross circulation continuing. 2: 0.5 γ histamine injected into a jugular vein of A. 3: the same dose injected in animal B.

Guinea-pig A: body-weight 360 g, histamine content of plasma 120 γ/1.  
Guinea-pig B: body-weight 340 g, histamine content of plasma 310 γ/1.  
Narcosis: 2 g urethane per kg of body-weight + 0.1 g of sodium phenylethyl barbituric acid subcutaneously.

system was filled with Tyrode solution containing heparine. Cross circulation was established at the moment when the clips were transferred from the tubings connecting the animals to those connecting the central and peripheral cannulae of each animal. The blood then flowed from one animal through the central cannula, the connecting tubing and the peripheral cannula of the other animal out into the branches of the carotid artery. After some minutes the cross circulation was interrupted. The histamine sensitivity of the bronchi was tested in each animal by intravenous injection of histamine. Unfortunately, in these experiments the anaesthesia, in order to prevent

spontaneous breathing, must be very deep, which reduces the histamine sensitivity.

In these experiments the plasma histamine concentration of about one hundred guinea-pigs was determined. The blood was withdrawn by heart puncture and plasma was ultrafiltrated previous to the assay on the gut. Two animals, one with a very low, the other with a very high plasma histamine value, but of about the same body weight were selected for each experiment. We found 9 such pairs having the following plasma histamine concentrations: 100—335, 120—270, 125—275, 130—300, 100—380, 175—265, 140—230, 140—350, 120—310 γ per litre. The actual experiments were always performed more than a week after the heart puncture.

In 6 out of the 9 experiments a marked bronchoconstriction was observed in the animal with the lower plasma histamine content. In 3 experiments no bronchoconstriction was elicited (the pairs 2, 3 and 4). In these 3 experiments the histamine sensitivity of the bronchi of the animal with the smaller plasma histamine content was found to be very low, while it was high in the 6 animals which reacted with bronchoconstriction. In none of the animals with the greater plasma histamine concentration was there a change of the bronchial tone, in spite of the fact that in some controls a high histamine sensitivity was revealed.

Figure 10 illustrates one of these experiments (pair 9). During cross circulation a bronchoconstriction was elicited in the animal with the low plasma histamine concentration. The histamine sensitivity of the bronchi was high in both animals.

The objection may be raised that it has not been proved that the bronchoconstriction in these experiments was caused by histamine. Yet it is remarkable that during cross circulation between an animal of a particularly great and another of a particularly small plasma histamine content a typical histamine effect is elicited in the latter; this occurs only if the histamine sensitivity is high. In the animal of great plasma histamine content no bronchoconstriction occurs even if the histamine sensitivity is high.

These experiments strongly support the conception, that histamine is present in the circulating plasma in an active state. There still remains the objection that an extremely labile compound might have been decomposed when the blood passed the system of cannulae and tubings; this assumption must, however, be rejected, since bronchoconstriction never occurred in the animal with the higher concentration of this hypothetical inactive compound in the plasma.

## Chapter 6

### Experiments where the histamine content of plasma is artificially increased

In these experiments we have raised the plasma histamine concentration by slow intravenous injection of histamine during a long period and have then tried to find out in what form this histamine appears in the circulating plasma. Using a special method we have been able to greatly augment the plasma histamine concentration in cats and dogs. Dale and Laidlaw (1919) found, that the intravenous injection of 100  $\gamma$  histamine per minute to a cat weighing 1.8 kg (56  $\gamma$  per kg and minute) caused a severe shock. Mc Carrell and Drinker (1941) showed, that in dogs a histamine quantity of the same order caused a decrease of the blood pressure of long duration. If, on the other hand, a smaller amount of histamine (about 1.5  $\gamma$ /kg and min.) is slowly injected into dogs the blood pressure first falls and then rises again in spite of continued injection; within 5—18 minutes the pressure returns to its original level (Giraud-Costa and Gayral 1940). In cats and dogs we have been able to confirm this and we have further made the following observation. After the original blood pressure has been re-established the slow injection can be continued with double the amount of histamine, the blood pressure then showing only a very transient fall. The dose can be doubled repeatedly, each increase in dose causing only a small or no fall in blood pressure. By this method it is possible to inject histamine in concentrations which would kill an untreated animal.

Many investigators have observed that the histamine sensitivity of the body can be diminished by previous histamine injections. Dale and

Laidlaw (1911) injected an amount of histamine into a cat which caused severe symptoms and found that the double amount elicited a considerably smaller reaction when given shortly afterwards. Similar observations have been made by Fuehner (1912), Oehme (1913), Eichler and Killian (1931), Müller et al. (1932). A decrease in histamine sensitivity of blood vessels after previous injections of histamine has been observed by several investigators (Phemister and Handy 1927, Epstein 1932, v. Euler 1938, Anrep et al. 1939, Heidemann 1943). These observations have been made in acute experiments. Other investigators have made experiments of a somewhat different type. They have tried to find out if the histamine sensitivity can be altered by a series of histamine injections, e.g. daily injections during a period (Strömbeck 1932, Schiff 1938, Farmer 1939, Edholm 1942, Katzenstein 1944). In this connexion it is also of interest that many investigators have tried to protect experimental animals against anaphylactic shock by histamine pretreatment (Smith 1939, Farmer 1939, Arloing et al. 1939); histamine injections have also been used in the treatment of allergic and other conditions, where histamine liberation is believed to play a part (Horton et al. 1936, Horton 1941, Rainey 1943, Butler and Thomas 1945).

Our investigation was carried out on cats under chloralose and dogs under morphine-chloralose. In some experiments artificial respiration was used. The blood pressure in the carotid or femoral artery was registered by a mercury manometer. Histamine biphosphate, dissolved in Tyrode solution, was slowly injected by a special device into a femoral vein. The solutions were of relatively high concentration so as to avoid the injection of great amounts of fluid (0.2—0.4 ml per minute was given). Separate injections were made through a cannula, inserted in the other femoral vein. From a cannula in a femoral artery 5 to 15 ml blood was collected in a centrifuging tube, containing heparine. Plasma was extracted or ultrafiltrated and then tested on guinea-pig's ileum.

The results of these experiments are summarized in tables 18 and 19. These experiments show that the histamine concentration of plasma can be raised to very high levels without a marked fall in blood pressure, provided that the high level is established successively. The injected histamine is rapidly eliminated. It is well known that injected histamine very rapidly disappears from the blood (Anrep and Barsoum 1935, Dragstedt and Mead 1935, Mac Intosh 1938, Rose and Browne

TABLE 18.

Nr	Injection of histamine			Histamine content of plasma		Blood pressure, mm Hg		
	Duration of injection, minutes	Total amount of histamine injected γ/kg	Largest dose of histamine injected γ/kg and min.	before injection	after injection	before injection	during collection of first sample after injection	
				γ/l	time after end of injection, min.			
1 cat	100	470	16	30	140 95 40	1 17 30	170	145
2 cat	50	750	30	60	150	0	—	—
3 cat	120	1850	15	30	205 160 100 100 50	0 7 16 35 85	150	150
4 cat	150	715	16	40	50	20	140	210
5 cat	50	500	10	40	130	0	160	135
6 cat	75	1125	15	40	180 170 45 40 45	0 6 50 85 100	140	125
7 cat	90	630	7	30	100 65 45 50 35 35	0 5 10 16 25 32	145	130

1938, Billings and Maegraith 1938, Code 1939, Hildebrand 1940).

Some of these experiments have been used for the identification of histamine in the ultrafiltrates as described in chapter 3. Our chief aim, however, was to investigate if the histamine, which can be detected in plasma after the injection, is present *in vivo* in a physiologically active state or has been inactivated

TABLE 19.

Nr	Injection of histamine			Histamine content of plasma			Blood pressure, mm Hg	
	Duration of injection, minutes	Total amount of histamine injected γ/kg	Largest dose of histamine injected γ/kg and min.	before injection γ/l	after injection γ/l	time after end of injection, min.	before injection	during collection of first sample after injection
8 cat	60	1940	250	50	1800	0	130	80
9 cat	50	750	15	25	120	0	200	160
10 cat	300	3925	200	—	2000 290 200 210 160 135 125	2 15 30 45 60 75 90	110	100
11 cat	230	2275	50	50	420 300 175 130 160 145	2 12 24 32 40 60	65	80
12 cat	185	5650	200	35	200	13	100	80
13 cat	320	7150	120	50	1000	2	—	—
14 cat	315	20650	560	—	14000 9500 7400	5 15 35	240	180
15 cat	285	18400	800	—	7800 2100 500 290 330	3 10 30 60 90	140	105
16 dog	80	975	50	60	300	17	140	110
17 dog	220	5900	200	65	4300 1600	0.5 7	—	110

by combination e.g. with some plasma component. The following three observations indicate that histamine in these experiments is present in plasma in a physiologically active state:

1) the plasma ultrafiltrate from blood, withdrawn after slow injection of histamine is found to contain the same amount of histamine as the plasma extract when tested on the gut. Table 20, in which the animals are numbered as in tables 18 and 19 gives the histamine concentrations of ultrafiltrates and extracts.

TABLE 20.

Nr	Histamine content of plasma, $\gamma/l$			
	before injection		after injection	
	extraction	ultrafiltration	extraction	ultrafiltration
2	60	—	150	150
7	30	45	100	100
8	50	60	1800	1850
9	25	25	120	130
10	—	—	—	2000
11	50	50	420	400
12	35	45	200	200
13	50	50	—	1000
14	—	—	1400	1550
15	—	—	—	7800
16	60	60	300	320
17	65	60	4300	4500

2) the more the plasma histamine concentration is increased, the smaller the fall in blood pressure becomes, caused by a dose of histamine intravenously injected. This fact is illustrated by figure 11. In this experiment, before the slow histamine injection, the plasma contained 30  $\gamma$  histamine per litre, and 0.3  $\gamma$  histamine, given intravenously, caused a fall in blood pressure of about 80 mm Hg. After the histamine content of the plasma had been raised to 140  $\gamma$  per litre, 0.3  $\gamma$  histamine caused a fall of only 20—30 mm. When the histamine concentration had decreased to 95  $\gamma$  per litre, the fall in blood pressure was about 50 mm, and when the histamine concentration had returned to nearly the original level the histamine sensitivity was almost restored.

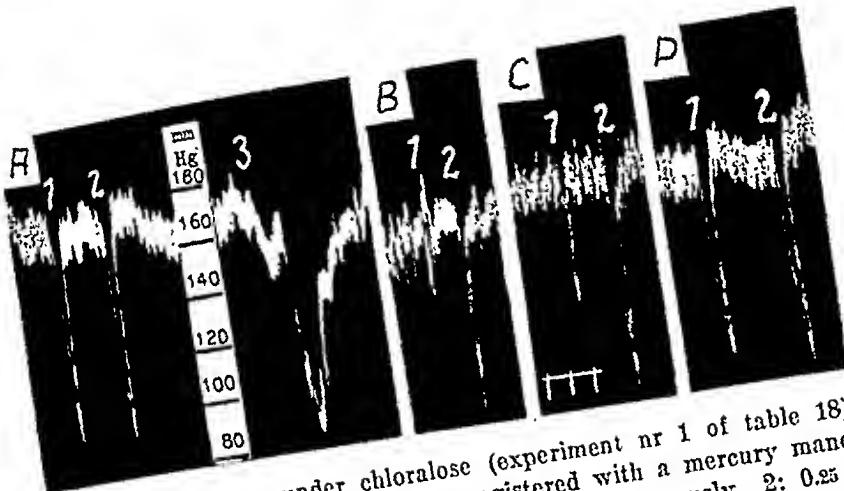


Fig. 11. Cat, 3.5 kg, under chloralose (experiment nr 1 of table 18). Blood pressure in the femoral artery registered with a mercury manometer. Time in minutes. 1: 0.30 γ histamine intravenously. 2: 0.25 γ acetylcholine. At 3 the slow injection of 1 γ histamine per kg and minute began. The dose was successively increased to 2, 4, 8, 16 γ per kg and minute between A and B for about 100 minutes. The injection was interrupted about 3 minutes before B. Between B and C there was an interval of 6 minutes, between C and D 13 minutes. Between A and C there were withdrawn, extracted and tested on guinea-pig's gut: Four plasma samples before A. Histamine content: 80 γ per litre. b) one minute after the end of the slow injection: 140 γ per litre. c) immediately after C: 95 γ per litre. d) immediately before D: 40 γ per litre.

These findings may have different explanations; yet it seems reasonable to conclude that the histamine in these experiments was present in plasma in a physiologically active state.

The experiments seem to be analogous to those of Barsoum and Gaddum (1935) who observed that the histamine sensitivity of an isolated piece of gut is highly diminished if the gut is suspended in a strong histamine solution. Our experiments may contribute to the discussion of the possibility to desensitize an organism to histamine, injected or intravittally liberated, by a pretreatment with histamine. Under our experimental conditions — acute experiments on cats under chloralose with the blood pressure as a test and histamine injected as described — the rule seems to be that the histamine injected is depressed only as long as the histamine content of plasma is increased.

3) if the histamine content of plasma is highly raised in a cat by means of slow histamine injection, the transfusion of blood to an untreated cat causes typical histamine effects in

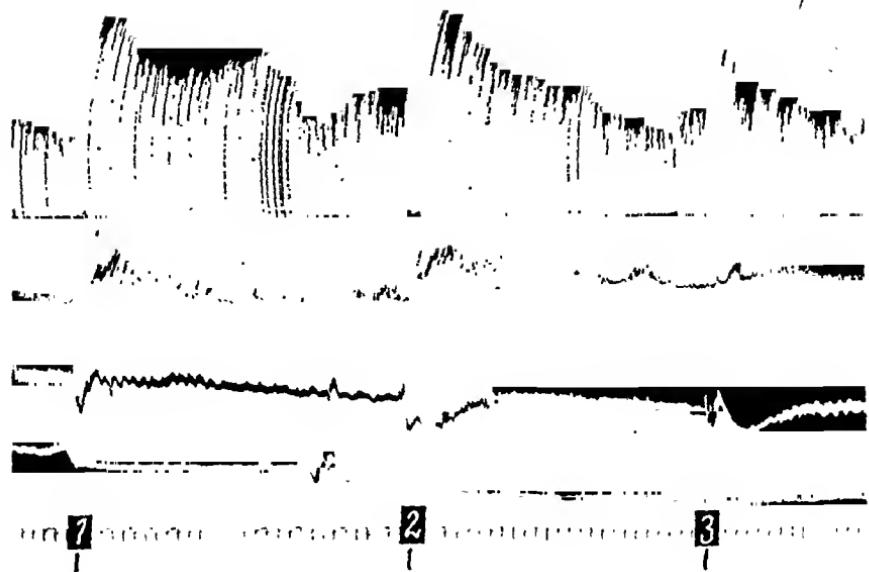


Fig. 12. Registrations from above: bronchial tone (according to Konzett and Rössler), jejunal motility (registered with a water manometer, connected with a balloon, inserted in the jejunum through an incision in the abdominal wall) and blood pressure in the femoral artery of the recipient cat B; blood pressure in the carotid artery of the donor cat A; time in minutes; signal marks. 1: transfusion of blood. 2: 100  $\gamma$  histamine to cat B. 3: 50  $\gamma$  histamine to cat B.

the recipient. Four such experiments were made giving similar results. Figure 12 illustrates such an experiment.

In a cat A weighing 3.3 kg (cat nr 15 of table 19) histamine was slowly injected in increasing doses (1.2 — 800  $\gamma$  per kg and minute) for nearly five hours. On another cat B (1.7 kg) the bronchial tone, the jejunal motility and the blood pressure were registered. A cannula, placed in the central stump of the femoral artery of cat A was connected by means of a piece of rubber tubing with another cannula inserted in the femoral vein of cat B. The connecting system was filled with Tyrode solution and the rubber tubing was occluded by a clip. Heparine was injected into both animals. Three minutes before 1 in figure 12 the injection of histamine into cat A was interrupted. At 1 the clip was opened for 25 seconds and the blood allowed to flow from cat A to cat B. As can be seen from the figure the transfer of blood from the

donor caused bronchial constriction, contraction of the gut and fall in blood pressure in the recipient. The cannula was then removed from the femoral vein of cat B. The slow injection of histamine to cat A was then repeated for a few minutes. Three minutes later the clip was again removed and blood allowed to flow through the transfusion system into a measure. 15 ml of blood was collected in 25 seconds. At 2, 100  $\gamma$  histamine dissolved in 15 ml warm Tyrode solution was injected intravenously for 25 seconds to cat B. At 3, 50  $\gamma$  histamine was given in the same way. The blood sample was used for determination of the hematocrit value of the blood and for estimation of the histamine content of the plasma on guinea-pig's gut. It was found that the blood contained 62 per cent of plasma and that the plasma contained 7800  $\gamma$  histamine per litre. Presuming that 15 ml blood containing 7800  $\gamma$  histamine per litre was transfused, cat B should then have received 70—75  $\gamma$  histamine. It can be seen in figure 12 that 50—100  $\gamma$  histamine, injected in a similar way, has an effect of the same order as the transfused blood. It is obvious that the figures obtained in this way are not very exact; it may be mentioned that the quantitative relations might be complicated e.g. by the fact that the blood of the donor probably had a relatively high concentration of adrenaline which might have interfered with the assay. Nevertheless the experiment indicates that most of the histamine, which was detected in the plasma of the donor (7800  $\gamma$  per litre), was present in a physiologically active state.

These series of experiments show, that histamine can be present in plasma of cat and dog in a concentration, which considerably exceeds the normal level without serious depression of blood pressure. In these experiments the histamine was not, at least not to a large extent, bound as an inactive compound; histamine occurred in plasma in a physiologically active form.

## Chapter 7

### Discussion

The discovery, that physiologically highly active substances such as acetylcholine and histamine are present in the body in high concentrations, introduced an interesting physiological problem. Best, Dale, Dudley and Thorpe (1927) in their paper on »the nature of the vasodilator constituents of certain tissue extracts» suggested two explanations. The active substance may exist »only potentially, in form of some inactive precursor» or it may be »present as such in the cell interior, being prevented from leaving it so long as the cell membrane is physiologically intact». Recently several workers have dealt with the problem of »free» and »bound» histamine and acetylcholine. In this connexion experiments by Abdon et al. (1939, 1944, 1945) are of special interest from which they conclude, that in the body there exists a labile complex compound, from which acetylcholine is very easily liberated but which does not itself exert the effects typical to acetylcholine. Many observations suggest that histamine is present in the cells in an inactive form, but there is so far no experimental evidence of the existence of a histamine precursor.

With the discovery, that histamine is a normal constituent of blood the question arose whether this histamine is physiologically active or inactive. Gaddum (1936) called attention to this problem. The problem was experimentally dealt with by Tarras-Wahlberg (1936).

In our experiments we have tried to find out if histamine is present in plasma in a physiologically active or inactive state. As is well-known histamine, present within intact blood cells,

does not exert any effects (Anrep et al. 1936, Barsoum and Smirk 1936). At the beginning of our experiments it seemed reasonable to assume that the plasma histamine was present in a »bound» state; considering the high physiological activity of histamine and also the histaminolytic power of tissues and plasma it is difficult to conceive that histamine can exist in plasma in a free state.

Our experiments have given the following results. In an ultrafiltrate of a plasma sample histamine is present in a physiologically active state. This is the case even if the ultrafiltrate is obtained by ultrafiltration *in vivo* when the blood is handled with the utmost care. By ultrafiltration approximately the same amount of histamine is recovered from a plasma sample as by chemical extraction. From these experiments it can be concluded that histamine is not present in plasma as a stable, inactive compound; that histamine should have been produced from histidine at the preparation of the plasma for the assay is out of the question. Kaiser (1939) is of the opinion, that the plasma histamine is adsorbed to protein and physiologically inactive; he believes that histamine, added to plasma, is rapidly inactivated by adsorption. The ability of plasma proteins to combine with other substances is well established and has been referred to as its »Vehikelfunktion». If for instance certain digitalis glucosides, such as digilanid A, are mixed with blood serum, they are partly adsorbed by albumen and their pharmacological activity is weakened (Suter 1944). Trypanblue, intravenously injected, is bound to proteins (Hechter 1943). A portion of the calcium, normally present in plasma occurs as a protein compound; if calcium is injected intravenously, part of this calcium combines with protein (Rona and Takahashi 1910, Cushny 1920, D'Silva 1934, Cameron and Moorhouse 1937). Histamine can, as a result of our experiments, not be present in plasma bound in a similar way. The three substances referred to above can not be detached from the proteins by ultrafiltration or dialysis, whereas the same amount of histamine by ultrafiltration can be recovered from plasma as by chemical extraction; this is applicable to the normally present as well as to injected histamine. There is also another difference. The fraction of the plasma

calcium, which is bound to protein, is not contained in the cerebrospinal fluid. Our experiments show, that histamine is present in the aqueous humour in about the same concentration as in plasma. The resemblance between aqueous and cerebro-spinal fluid as to mode of formation and composition has often been stressed.

Our experiments with ultrafiltration in vitro and in vivo and with aqueous humour indicate, that if histamine is normally present in plasma as a physiologically inactive compound, this compound must be extremely labile. The cross circulation experiments strongly support the view that histamine exists in circulating plasma in a physiologically active form. It is also demonstrated that intravenously injected histamine can circulate for some time in an active state in the plasma.

We have considered it necessary to make two kinds of control experiments. Firstly we have tried to ascertain that the active substance found in plasma samples really is histamine. In a physico-chemical respect the substance corresponds to histamine in being ultrafiltrable and not destroyed by Code's extraction procedure. As to the biological properties we have found a close agreement. The substance contracts the isolated guinea-pig's gut; this effect, like that of histamine, is antagonized by thymoxyethyldiethylamine or theamine. It depresses the blood pressure of the atropinized cat when injected intravenously; on close arterial injection in the adrenals, the agent, like histamine, causes a rise in blood pressure. The ultrafiltrate does not give a fall in blood pressure in a cat which has previously been treated with histamine. It constricts isolated guinea-pig's bronchi. The identity between the agent of the ultrafiltrate and histamine is also suggested by quantitative relations: both have the same concentration-action curve on isolated guinea-pig's gut; when matching the ultrafiltrate against histamine equal results are obtained on different test objects. The ultrafiltrate has no significant effect on the rabbit's blood pressure and other test objects not affected by histamine in moderate concentrations. The active agent is identical with histamine in every respect investigated; no substance other than histamine is known, which has all the properties described here.

We have further tried to ascertain that histamine really is a normal constituent of the circulating plasma and that the histamine found in a plasma sample, *in vitro*, has not been liberated from the corpuscles when securing the sample. The experiments on rabbit's blood show, that clotting or rough handling of the blood can cause a considerable increase of the plasma histamine concentration; a carefull treatment of the blood, on the other hand, and especially ultrafiltration *in vivo* gives samples with low histamine concentration. Yet many of our observations support the assumption that histamine is normally present in the circulating plasma: in guinea-pigs, rats and dogs the plasma histamine concentration is maintained at a fairly constant level during long periods; histamine is present in *in-vivo*-ultrafiltrates from dogs and rabbits and in aqueous humour from guinea-pigs, rats, rabbits, cats and dogs; in the species investigated the histamine concentration of plasma *in vitro*, aqueous and *in-vivo*-ultrafiltrate is of the same order. The plasma samples from rabbits are quite an exception, but there is reason to believe that also in rabbits histamine is present in the circulating plasma in the same concentration as in aqueous and *in-vivo*-ultrafiltrate. The cross circulation experiments in guinea-pigs strongly suggest that histamine is really contained in the circulating plasma.

Summarizing all our experimental evidence it can be concluded that histamine is really present in the circulating plasma in a physiologically active state.

Our experiments may contribute to the understanding of the puzzling fact, that this highly active substance can be present in plasma. From the experiments in chapter 6 it is obvious that the circulatory system has a considerable adaptability to high plasma concentrations of physiologically active histamine, provided that the concentration is increased slowly. In these experiments the histamine was injected in gradually increasing doses, but apart from this no measures were taken which could justify the assumption that an adaptation to histamine should be possible only under these special experimental conditions. On the contrary, it must be remembered that in experiments of this type anaesthesia and loss of blood (due to withdrawal of

blood samples) greatly decrease the tolerance to histamine (Dale 1920). We have not investigated the mechanisms which maintain the blood pressure at a high level in these experiments. Reflex mechanisms should be involved. Apart from that, it seems likely that the effector cells can adapt themselves to high histamine concentrations just as the cells of the isolated gut, bathed in a strong histamine solution. Besides, antagonistic substances may counteract the effect of histamine present in plasma. Histamine causes an output of adrenaline from the suprarenals; a physiological antagonism between these two substances has been discussed (Dale and Richards 1918, Burn and Dale 1926). Other substances may also play a part; Ackermann and Wasmuth (1939) suggest, that the antagonism between histamine and arginine or histidine may be of physiological significance.

If the conception that active histamine is present in plasma is accepted, another problem arises, which has in our introduction also been referred to: How is it possible that histamine can exist in plasma, where it is subjected to a histaminolytic activity? It should, however, be stressed that our experiments only indicate that histamine is present in an *active* state; they do not prove that it occurs in a *free* form. The possibility still remains that the plasma histamine is active, but bound as a compound where it is protected against the histaminolytic activity. Dale (1932) suggested that acetylcholine might exist in an active complex compound, in which it can not be attacked by the acetylcholine esterase. That histamine could exist in a bound state and yet be physiologically active has also been suggested by Dale (1937—38); in such a hypothetical compound it seems necessary that two groups of the histamine molecule should be free: the NH<sub>2</sub>-group, to which the specific activity is due, and the NH-group, by which the histamine is anchored to the tissues (Rocha e Silva 1944). It may be added that Sachs et al. (1932) have observed, that histamine can exist in an adsorbed state and yet have a depressant and secretory activity.

The problem under discussion can also be explained in another way. It may be possible that histamine is permanently destroyed and that there is at the same time a continued inflow of his-

mine into the blood. This histamine may originate from metabolic processes of the tissues (see e. g. Burn and Dale 1926, Dale 1927, 1929, 1933, Lewis 1927, Gotzl and Dragstedt 1940), or it may be derived from the food, possibly produced by bakteria in the gut (Mellanby and Twort 1912, Abel and Kubota 1919, Koessler and Hanke 1924). If this be true there must exist some regulating mechanism which effectively balances inflow and elimination of histamine. The fact that the histamine concentration in one and the same animal is maintained fairly constant for a long period and that, after histamine injections, the concentration rapidly returns to the original level, indicates that such a regulating mechanism really exists.

The finding that histamine is present in plasma in an active form gives rise to the question if this histamine has any physiological function. On this point nothing is known with certainty, and although such functions may come within many domains it is tempting to refer to the hypothesis of a »balanced chemical control of capillary tone» in which histamine is believed to play a part (Dale and Richards 1918, Burn and Dale 1926).

## Summary

Experiments have been carried out on guinea-pigs, rats, rabbits, cats and dogs.

1. Ultrafiltrates of plasma from these animals contain an agent which contracts the isolated, atropinized guinea-pig's ileum; this effect is antagonized by thymoxyethylethylamine or theamine. The activities of the ultrafiltrate and of histamine follow the same concentration-action curve. The plasma ultrafiltrate depresses the atropinized cat's blood pressure when injected intravenously; on close arterial injection in the adrenals it raises the blood pressure. The bronchi of the perfused guinea-pig's lung are constricted by the ultrafiltrate. It is concluded, that ultrafiltrates of plasma contain an agent which is identical with histamine.

2. The histamine concentration of plasma from guinea-pigs, rats and dogs is maintained at a fairly constant level during long periods. This indicates that histamine is a normal constituent of plasma and that the histamine present in plasma samples *in vitro* has not been liberated from the blood corpuscles. This conception is further supported by the observations mentioned below.

3. A plasma ultrafiltrate contains histamine in about the same concentration as a corresponding sample, extracted chemically. The histamine content of aqueous humour and of *in-vivo*-ultrafiltrates is approximately the same as that of plasma. From these findings it is concluded that histamine exists in plasma in a physiologically active form or as an inactive compound, from which histamine is extremely easily liberated. Cross circulation experiments on guinea-pigs indicate that histamine is present in an active form in the circulating plasma.

4. By slow intravenous injection of histamine at increasing rates the histamine concentration of the plasma can be considerably raised in cats without any serious depression of the blood pressure. It is concluded that histamine, injected in this way, is carried by the plasma in an active form.

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Stockholm*

A STUDY OF THE RESPIRATORY  
REFLEXES ELICITED  
FROM THE AORTIC AND  
CAROTID BODIES

By

BO E. GERNANDT

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*Stockholm 1946*



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## PREFACE

The present work has been carried out at the Physiological Department, Karolinska Institutet, where for many years particular interest has been devoted to the reflexogenie effect under various conditions upon the respiration and circulation elicited from the carotid sinus region. It is in this connection a great pleasure for me to express my warm thanks to the head of this department, Professor ULF VON EULER, who aroused my interest in the first place for physiological research. In the course of the work he has always discussed with me the problems arising with great willingness and interest, and here his long experience in this field has been of great help to me.

To Professor YNGVE ZOTTERMAN, with whom during the past years I have had the great privilege of cooperating, and from whom during this period I have learned the electro-physiological technique, it is a sincere pleasure for me to express my deep gratitude for all valuable advice as well as criticism and encouragement in connection with this work. I am also much indebted to him for placing his laboratory and apparatus at my disposal.

For the assistance which my wife has always so readily given me, and which has been a great time-saving help, I will here express my warm thanks.

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B O E. G E R N A N D T

## INTRODUCTION

Since the discovery of the chemoreceptors in the carotid sinus region by HEYMANS, BOUCKAERT and DAUTREBANDE (1930) these have been made the object of investigation by a very large number of physiologists, so that their function has now been made clear in many essential respects. Even several years earlier, however, J. F. HEYMANS and C. HEYMANS (1927) had shown the existence and importance of such receptors in the region around the beginning of the aorta. As these chemoreceptors, as compared with those localized to the carotid sinus, are of more subordinate importance and do not, moreover, from the technical point of view, offer such favourable experimental conditions, they have been less sought after for special physiological studies. In experiments on the reflex effect from the chemoreceptors on respiration, blood pressure, pulse frequency, width of vessels, adrenaline secretion etc., most writers, taking as their point of departure the fundamental investigations of HEYMANS and HEYMANS, have only had the chemoreceptors of the aorta in mind, but the main interest has been directed to the receptors in the carotid bodies. Only COMROE (1939) has made the chemoreceptors of the aorta the object of renewed direct investigation.

Long before the attention of the physiologists had been directed to the problems in this particular field and before they had been able to provide an explanation for them, the morphologists knew of the occurrence of so-called paraganglionic formations in these regions.

The intention behind the present investigation has been with the aid chiefly of electro-physiological recording of the impulses in the nerves from the chemoreceptors of the aorta to study more closely the effect of chemical changes in the blood on the inflow of afferent impulses. Also the effect of drugs having a specific stimulating effect on the chemoreceptors has been studied.

By means of a method of selectively eliminating the chemical reflexes from the aorta and sinus regions, while the pressor reflexes were left intact, a different approach to problems which have been the subject of diverging opinions was made possible. This procedure makes it possible to leave the buffer nerves regulating the blood pressure, the sinus and depressor nerves, intact. Previously, in the study of the effect reflexly elicited from the chemoreceptors, a selective elimination of the chemoreceptors of the aorta has not, apart from the denervation procedure, been practised. On the other hand, it has been possible mechanically to destroy the chemoreceptors in the carotid sinus while the in other respects intact sinus nerve still conducted pressor impulses. The beyond all comparison most common method, however, has been to sever the sinus and depressor nerves (or the vago-depressors), in order to exclude the effect from the chemoreceptors. In consequence of the fact that the buffer nerves had then been eliminated, the blood pressure has shown such changes that the effects investigated have been influenced, and a pure picture of the actual mechanism has thus not been obtained.

After selective elimination in order of the chemoreceptors of the aorta and sinus regions, it has been possible to investigate the distribution of the reflexly stimulating effect over the respective chemoreceptors on specific stimuli. The eventual inhibiting effect on the respiration released from the baroceptors has also been made the object of special interest.

## I. The existence of extra-carotid chemoreceptors

In a work published in 1927 J. F. HEYMANS and C. HEYMANS were the first to show that the respiration could be affected reflexly from the thoracic region, in which connection chemical stimuli served as the eliciting factor. The effect also appeared, as the authors were able to show, if one only perfused the heart and the nearest part of the aorta of dog, the head being isolated from the body with the exception of both vago-depressors. An increase of the respiratory movements of the head was brought about when the content of carbon dioxide in the blood was increased or if its oxygen-content was reduced. The only path the respiration-stimulating impulses could take was over the vago-depressors, and it was thus shown that chemical changes in the blood could elicit respiratory reactions by means of reflexes elicited from structures outside the respiratory centre. For the chemical regulation of the respiration was formerly considered to take place exclusively over the respiratory centre in the medulla oblongata, but through the investigations of the above authors these problems appeared in a totally different light, interest in these questions was immensely stimulated, and in the course of the years an extremely copious literature on the subject has seen the light.

HEYMANS, BOUCKAERT and DAUTREBANDE (1930, 1931) were afterwards able to show that analogous conditions existed also in the carotid sinuses. In these experiments on dog, the in respect of the circulation isolated sinus region was perfused with the aid of another animal or by means of a pump, whereby all central effects could be excluded. That it was possible to provoke respiratory reactions from these regions through endosinusal alternations of pressure had already been shown by SICILIANO (1900),

PAGANO (1900), MOISSEJEFF (1927), HEYMANS (1928), HEYMANS and BOUCKAERT (1930). Through the investigations carried out by HEYMANS and his co-workers it could be shown that hypoxemia does not directly affect the respiratory centre, but acts almost exclusively reflexly over the sinus and aorta regions. Of these two reflexogenic zones, the first is by far the more important, as is shown by the fact that only denervation of the sinus regions inhibits the stimulating effect of hypoxemia upon the respiration almost completely. Carbon dioxide, on the other hand, still has a stimulating effect on the respiration after a severing of the nerve connections from the aorta and sinus regions, so that we must assume that carbon dioxide has an in the main direct effect upon the respiratory centre.

As has already been mentioned, the localization and the great physiological significance of the chemieal receptor fields in the carotid sinuses have engaged the interest of the majority of writers in this sphere, while a comparatively small number, on the other hand, have devoted a closer study to the reflexogenic, chemosensitive aorta zone. Also the occurrence of chemical receptor fields in the aorta has been questioned by some writers. BEYNE, GAUTRELET and HALPERN (1933) carried out experiments on dogs anaesthetized with chloralose and enclosed in a chamber in which the atmospheric pressure was then lowered. While the animals could easily adapt themselves to the ventilation magnitude at a sub-pressure corresponding to 8000—9300 meters, this was not possible after bilateral severance of the nerve connections to the carotid sinuses. Cyanosis then appeared rapidly at a pressure corresponding to 3000—4000 meters, with cessation of the respiration and death. The authors therefore denied that anoxemia could exert any stimulus on the respiration otherwise than over the sinuses.

On careful denervation of the carotid sinuses in dogs, cats and rabbits it sometimes happens that the respiration ceases and the animal dies (WITT, KATZ and KOHN 1934, GEMMILL, GEILING and REEVES 1934). EULER and LILJESTRAND (1936) as well as DAUTREBANDE and WEGRIA (1937) found in dogs and cats anaesthetized with chloralose that in the reflex effect on the

respiratory centre in connection with hypoxemia the carotid sinuses play the main rôle, whereas the region that is innervated by the depressor nerves is practically insensitive to hypoxemia.

Other writers, again, have confirmed the existence of extra-carotid chemoreceptors by showing that even after complete sinus denervation hypoxemia still causes a slight increase of the respiration, though this disappears when the vago-depressors are severed. SELLADURAI and WRIGHT (1932 a) studied the effect upon the respiration at various oxygen concentrations on cats that had been decerebrated or anaesthetized with chloralose, and found that the respiration-stimulating effect of the hypoxemia was entirely dependent upon the buffer nerves (the depressor nerves, the sinus nerves), but that the latter are of far greater importance than the afferent fibres in the depressor nerves. SCHMIDT (1932) arrived at the same result in experiments on dogs and cats, but not on rabbits, in which he could not find any release of reflexes from the aorta in connection with hypoxemia. JONGBLOED (1936) studied the regulation of the respiration in dogs in connection with hypoxemia that had been provoked by lowering the atmospheric pressure in a chamber. He found that after both the depressor nerves and the sinus nerves had been severed there was still a certain regulation of the respiration, though this disappeared entirely if also the vagi were severed. This may be explained by the fact that depressor fibres also run in the vagus (Koch 1931). After severance of the sinus nerves and the vago-depressors (as a rule already after severance of only the sinus nerves) hypoxemia no longer stimulates to increased respiration, but has rather a paralytic effect. These peripheral chemoreceptors in the carotid sinuses and the aorta thus to some extent protect the respiratory centre and thereby the entire body from the consequences of hypoxemia. Also GESELL and MOYER (1937) observed the existence of chemoreceptors in the aorta of dog in experiments on hypoxemia. LAMBERT and GELLIHORN (1938) studied especially the effect of hypoxemia on the blood pressure in narcotized dogs with pneumothorax and constant artificial respiration. They gave gas mixtures poor in oxygen that in the intact animals caused a rise in the blood pressure, but that in animals

in which the sinuses had been denervated and the vago-depressors severed produced a lowering of the blood pressure. On a closer analysis of how the effect was localized to the respective buffer nerves the authors found that only the severance of the one pair of buffer nerves did not cause a lowering of the blood pressure in connection with hypoxemia. These experiments thus indicate that the heightening effect of hypoxemia on the blood pressure is dependent upon the chemoreceptors both in the carotid sinuses and in the aorta region. COMROE (1939) carried out a physiological and anatomical investigation on the significance and the localization of the chemoreceptors of the aorta in dog and cat.

The existence of other chemoreceptors than those in the carotid sinuses and at the beginning of the aorta with any physiological significance has not been demonstrated, despite the fact that there exist so-called paraganglia with an appearance similar to these in other places in the body.

## II. Anatomical survey

### The aortic and pulmonary paraganglia.

The occurrence of limited cell groups of a paraganglionic nature in the tract around the aorta and arteria pulmonalis has long been known, and has been made the object of detailed studies by a number of morphologists. By paraganglion is meant a cell formation which may be of either sympathetic or parasympathetic nature. The former are developed from sympathetic, the latter from parasympathetic neuroblasts. Also mixed paraganglia occur. The existence of similar paraganglia in the carotid sinus region has also long been known, but through HERING's (1924) discovery of the sinus nerves attention was once more directed to this region, and a large number of researchers (DRÜNER, 1925, DE CASTRO, 1926, 1927, RIEGELE, 1928, SUNDER-PLOSSMANN, 1930, BOYD, 1937, and others) made it the object of investigations. Through the experimental investigations carried out by HEYMANS and HEYMANS (1927) and HEYMANS et al. (1930) data were obtained concerning the function of these formations, that had previously only been the object of guessing and free speculations, and also the physiologists had their attention drawn to problems in this field.

WIESEL (1906) described in connection with a morphological investigation of the heart in children the occurrence of a limited, greyish red formation embedded in the epicardial adipose tissue around the left coronary artery where this runs medially to and behind the left auricle. It proved to consist of typical chromaffin cells. Also TRINCI (1907), in an investigation on mammals and reptiles, observed the occurrence of chromaffin tissue around the base of the heart. BUSACHI (1912) was able in two fully developed embryos to distinguish two isolated groups of cells, an upper group localized just below the aortic arch and a lower one, that already found by Wiesel around the coronary artery. The upper group was characterized by deficient chromaffinity, the lower, consisting of chromaffin cells, was not to be found in adult persons. RABL (1922) found in embryo of the guinea-pig similar chromaffin formations laterally to the common carotid artery, between the vessel and cervical sympathetic trunk. Especially on the right side they occurred in large numbers, extending there down to the base of the heart. He suggested the designation *paraganglion caroticum inferius*, in conformity with the nomenclature introduced by KOHN (1900) ("Nebenorgane des peripherischen Nervensystems").

WATZKA (1930) and PENITSCHKA (1930, 1931) revived with their investigations interest in these cell groups of a paraganglionic nature. The latter studied especially the paraganglion, which was situated below the arch of the aorta in connective tissue between the aorta and the pulmonalis (Busachi). To this formation, which was characterized by deficient chromaffinity and which he found regularly both in man and in mammals, the author gave the name *paraganglion aorticum supracardiale*. It consisted of a cell-rich tissue that was in part embedded in a rich network of vagal and sympathetic fibres, where isolated ganglion cells also occurred, and the cell groups lay in part close beside the nerve branches, yet seemed always to be connected to the nerves and through these to be bound up into an organic unit. He pointed out the morphological agreement between this paraganglion and "paraganglion caroticum", although the former is smaller in size, and also mentioned that these two formations are situated in regions from which the afferent regulators of the blood pressure (the depressor nerve and Hering's nerve) proceed. They differ from the sympathetic paraganglia (*paraganglion suprarenale*, p. *aorticum abdominale*) in that they do not consist of chromaffin cells and do not produce adrenaline. The terms "paraganglionic" and "chromaffin" have thus not the same meaning, and paraganglionic has a wider application, for the paraganglia may consist of chromaffin (adrenaline-containing) cells or of non-chromaffin cells (not adrenaline-containing) or again of a mixture of these two kinds of cells. The sympathetic paraganglia disappears also in older persons, with the exception of the suprarenal medulla (ZUCKERKANDL 1901, IWANOFF 1925), which is not, however, the case with *paraganglion aorticum supracardiale* and "paraganglion caroticum". The former are supplied through sympathetic, but the latter are innervated in part through sympathetic but in part also through the cerebral nerves. The paraganglion described by Wiesel must therefore be considered to be of sympathetic nature.

PALME (1934) carried out a careful histological investigation of the paraganglia of the heart, and found that these, as Busachi had already pointed out, could be divided into two groups, and he proposed for them the designation *paraganglion supracardiale superius* (Busachi, Penitschka) and *inferius* (Wiesel, Trinci). In the cat he did not find these two easily distinguishable paraganglia, there was here a more diffuse distribution of paraganglionic tissue, which extended along the aorta from the point of departure of the left coronary artery and upwards to the egress of the large vessels and to the point of attachment of ligamentum Botalli, and along pulmonalis from its commencement to its point of bifurcation. It was, however, possible to observe a certain regularity in the localization in so far as in definite places, namely, under the aortic arch behind ligamentum Botalli, as well as just under the right pulmonary artery in the connective tissue between the aorta and pulmonalis and also, finally, at the base of the heart between the two main vessels, there were large groupings of cells. The paraganglion situated under the arch of the aorta did not contain any

chromaffin cells. The other paraganglia, on the other hand, are mixed, but with mainly chromaffin tissue, and these are found only in some full-grown cats. A wealth of nerves run around these paraganglia. Very often paraganglionic cell-islands are enclosed in the nerves. In the aortic arch around ligamentum Botalli and at the point of bifurcation of the pulmonary artery the author found a remarkable wealth of vasa vasorum, which ran into the media and which in the course of their passage through the adventitia sometimes received thin nerves containing paraganglionic cells. The adventitia of the aorta and pulmonalis are very rich in these nerves with paraganglionic cells. Sometimes one also finds them in the outer layers of the media. He assumed that they constituted the terminal branches of nervus depressor, which extends right into the media (KOESTER and TSCHERMAK 1902). In newborn cats he found that the upper paraganglion described by him sometimes received its blood supply through a small vessel proceeding from pulmonalis. A similar arrangement could sometimes be found in human embryos.

SETO (1935), in an investigation carried out on adult human beings, also found permanent paraganglionic tissue at the commencement of the aorta as well as the paraganglia between the aorta and the pulmonary artery, which he considered to receive a mainly parasympathetic innervation and only in a lesser degree sympathetic. This innervation agrees with the investigations of other writers on glomus caroticum (DE CASTRO, 1926, RIEGELE, 1928, MEIJLING, 1938). He considered that the afferent impulses from these formations must be conducted in the depressor nerve, which, as is known, is characterized by marrow-containing, centripetal fibres and has its region of distribution in the tract for these paraganglia. He pointed out, further, how each paraganglion lies close to a fairly strong main artery, or that such an artery runs to the middle of it. The main artery divides in the paraganglia and sends branches between the groups of cells to end, finally, in a number of capillaries that surround the parenchymal cells. As was the case with the arteries in the paraganglia of the carotids (DE CASTRO, 1927, RIEGELE, 1928), also the vessels in these cardiae paraganglia showed a large number of sensitive nerve-endings, which here doubtless derive from the depressor nerve.

MURATORI (1934, 1935) described the occurrence in mammals of other than the paraganglia mentioned here above, which were situated, on the right side, in the angle between the subclavian artery and the common carotid artery and on the left side just above the aortic arch medially to the left subclavian artery.

This discovery has been verified by NONIDEZ (1935), who observed in rabbits and cats, in the places indicated by Muratori, the existence of this paired organ, that consisted of two groups of round, non-chromaffin cells. These organs were enclosed in a thin capsule of connective tissue. Exactly similar cells were also found scattered around the endings of nervus depressor on the corresponding side, and sometimes also embedded in the adventitia of the aorta. The endings of the depressor nerve are found here in the adventitia,

Figures cor- responding to those in fig. 1	Author	Localization	Object of investig- ation	Name
1	WIESEL (1906)	Around point of departure for left coronary artery	Homo (child)	—
2	TRINCI (1907)	Around base of heart	Mammals Reptiles	—
3	BUSACHI (1912)	a) Just below aortic arch b) Around left coronary artery	Homo (full-time embryos)	—
4	RABL (1922)	Laterally to the common carotid artery and down to- wards the base of the heart	Embryo of guineapig	Paraganglion caroticum inferius
5	PENITSCHKA (1930, 1931)	Below aortic arch between aorta and pulmonalis	Homo Mammals	Paraganglion aorticum supracardiale
6	PALME (1934)	a) Below aortic arch between aorta and pulmonalis b) At beginning of left coronary artery	Homo (embryo) Cats	Paraganglion supracardiale superius Paraganglion supracardiale inferius
7	SETO (1935)	a) Below aortic arch between aorta and pulmonalis b) At beginning of aorta	Homo	— —
8	MURATORI (1934, 1935)	a) In angle between right subclavian artery and common carotid artery b) Just above aortic arch medially to left sub- clavian artery	Mammals	— —
9	NONIDEZ (1935)	a) In angle between right subclavian artery and common carotid artery b) Just above aortic arch medially to left sub- clavian artery	Rabbits Cats	Glonius aorticum
10	BOYD (1937)	a) At brachiocephalic trunk just under point of departure for right sub- clavian artery b) On ventral side of aortic arch somewhat below the point of departure for left subclavian artery	Homo Dogs	—
11	NONIDEZ (1937)			—
12	NONIDEZ (1937)	Below aortic arch between aorta and pulmonalis	Dogs	Aortic body

Tab. 1. The table gives in summary form the paraganglia described in detail in the text.

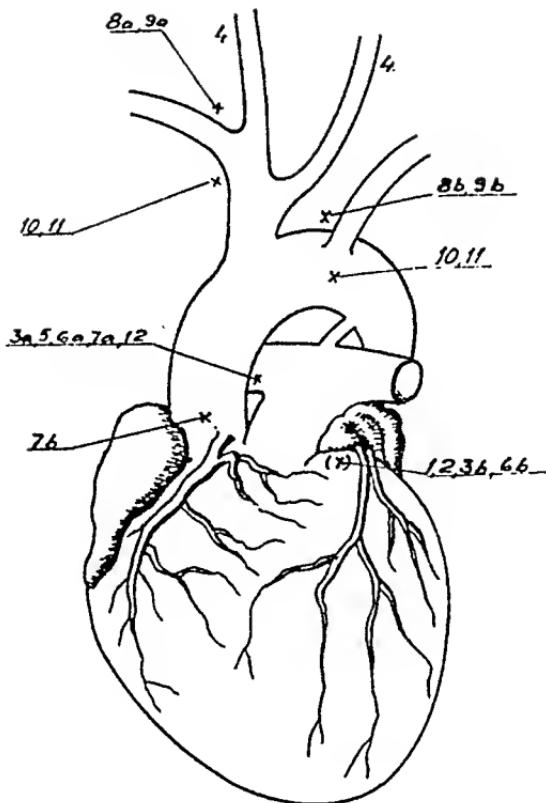


Fig. 1. Schematic drawing of the localization of so-called paraganglia around the aorta and the pulmonary artery. For more detailed description see the text and Tab. 1.

but also in the media of the right subclavian artery and the arch of the aorta, thus corresponding to the site for the above-mentioned formations (TSCHERN-JACHWWSKY, 1929). For this organ he introduced the term *glomus aorticum*. He also points out that both the depressor nerves receive sympathetic fibres mainly from ganglion cervicale inferius. That the depressor nerve contains sympathetic fibres has long been observed (SCHUMACHER, 1902, TELLO, 1923, and others). He found that the right glomus aorticum obtained its blood supply through a small artery proceeding from the corresponding subclavian artery or from the brachiocephalic trunk. The left glomus aorticum was supplied by an artery proceeding directly from the aortic arch or from the left subclavian artery. These arteries contain in their walls a plentiful supply of nerve-endings of baroceptor type.

In a later work by NONIDEZ (1936) he found in the kitten the small artery already described by Palme which supplies paraganglion aorticum supra-

cardiale (Penitschka) and proceeds from somewhat varying parts of the pulmonary trunk or from the right pulmonary artery, though as a rule from the medial side of the trunk and supplies the paraganglion with venous blood. In consideration of this blood supply the author suggested that the designation *glomus pulmonalis* should be introduced instead. He pointed out that it also probably received blood from the aorta or its branches.

HOLLINSHEAD (1939) studied the nerve supply to glomi aortici in degeneration experiments on cats, and found that the nerve fibres to glomi came entirely from cells localized in ganglion nodosum. When ganglion cervicale superius was removed by operation the author could not find any noticeable change in the innervation.

NONIDEZ (1937) found that the localization of the paraganglia in dogs differed from the localization in rabbits and cats. On the right side glomus aorticum consisted of an extremely small group of epithelioid cells embedded in the adventitia of the brachiocephalic trunk just under the point of departure for the right subclavian artery. Thus the site for the paraganglionic tissue in the human embryo described by BOYD (1937). On the ventral side of the aortic arch somewhat below the point of departure of the left subclavian artery the left glomus aorticum is situated. It receives its blood supply through a small artery proceeding from the ventral side of the aortic arch. Branches from this artery also reach down to similar groups of epithelioid cells situated on the ventral side of the place of bifurcation of the pulmonary artery at the base of ductus arteriosus. In this artery there are nerve-endings of baroceptor type. Such regions with baroceptors have also been demonstrated in vena cava and in vena pulmonalis by NONIDEZ (1937). Here, however, there are no paraganglia connected with them. Also in the space between the aortic arch and pulmonalis the author described in the dog the occurrence of similar cell groupings, which probably correspond to PENITSCHKA's paraganglion aorticum supracardiale in man. For this paraganglion he introduced the designation *aortic body*. It was supplied with blood through an artery which proceeded from the dorsal side of the aortic arch. A further group of cells of this kind lay between aorta ascendens and pulmonalis, and corresponded to Palme's paraganglion supracardiale inferius. They obtained blood through a branch of the left coronary artery. COMROE (1939) was unable to find this group of cells in adult dogs. The artery described in an earlier work of NONIDEZ (1936), which in the kitten proceeded from truncus pulmonalis and supplied the paraganglia between the aortic arch and pulmonalis, was not to be found in the dog, and is thus presumably not to be considered as of any fundamental importance, and the author accordingly abandons his previous opinion. In a work by GOORMAGHTIGH and PANNIER (1936) it was pointed out that this artery is

only to be found in the embryo of the cat and in the kitten, but is obliterated in the full-grown animals, in whom this paraganglion is thus supplied with blood through the coronary vessels or aorta ascendens.

In connection with serial sections ADDISON and COMROE (1938) found that in dogs the paraganglionic grouping of cells described by Penitschka and situated in the adventitia of the aorta obtained its blood supply through a small vessel proceeding from the aorta on a level with the brachiocephalic orifice.

The approximate positions of the paraganglia are marked in a schematic drawing (Fig. 1).

# AUTHOR'S OWN INVESTIGATIONS

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## III. Aortic body

### 1. Technique and Procedure.

Cats were used in most of the experiments but in some cases rabbits were employed. The cats were anaesthetized with 0.05—0.07 g of chloralose per kilogram of body-weight. The animals were first anaesthetized with ether, after which chloralose was injected intravenously in a 1 % solution. The rabbits were anaesthetized with urethane, 1.4 g per kilogram of body-weight being injected intravenously in a 20 % solution.

A tracheal cannula was introduced in the usual way. The blood pressure was recorded from the femoral artery by means of an Hg-manometer. Intravenous injection was given through the femoral vein.

In these experiments the respiration was recorded with an ordinary Marcy pneumograph connected with a Marey tambour.

For local, intra-arterial or intracardial injection a fine ureteral catheter was employed, which was moistened with glycerin to ensure an easier passage through the vessels. The catheter was generally introduced into the circulatory system through the right common carotid artery in the neck. In some cases it was introduced via the right or the left subclavian artery. By pushing the catheter down carefully it was possible to get the orifice of the catheter to lie in the spot desired. After every experiment the exact position was determined on autopsy. In order to get the catheter to glide through aorta ascendens it proved best,

owing to the anatomical conditions, to twist the animal, which was lying on its back, hard to the left. The passage past the aortic valves was recognized by a slight resistance, which was easily overcome. It was not possible to observe any considerable tearing of the valves. Different substances could then be injected through the catheter (lobeline, piperidine etc.).

In those cases in which a recording of the action potentials from the depressor nerve was carried out the dissection procedure was as follows. In cats the skin and musculature were dissected away over the clavicle and the three or four uppermost ribs (generally on the right side), which were severed. If the intercostal arteries and the internal mammary artery were ligated this operation could be carried out without any bleeding worth mentioning. After this the depressor nerve was sought at its point of entry into vagus, which was in the tract under the clavicle, and dissected out in a caudal direction. This was done under a magnifying glass, care being taken to see that the nerve was not stretched or pinched. The nerve was severed as far cranially as possible, and in order to increase the signal-to-noise ratio the nerve-sheath was carefully drawn off. To prevent the nerve from drying, it was kept moist with Ringer's solution at body-temperature, which was dropped onto it with a fine pipette. During the registration the animal was, moreover, placed in a moist chamber.

The recording of the action potentials was made by means of a capacity-resistance coupled amplifier and a cathode ray oscillosograph. This amplifying set allowed an amplification of up to  $10^7$  times, and thus enabled me to record the action potentials from very thin nerve fibres, provided the nerve preparation between the electrodes was of a small diameter, thus offering a high signal-to-noise ratio (see ZOTTERMAN 1936). The potentials were led off by means of Ag-AgCl-electrodes of the conventional type.

In the experiments on cats where the action potentials were recorded one was compelled, since the thorax was open, to give the animal artificial respiration by means of a Starling pump.

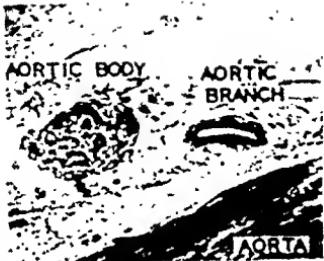
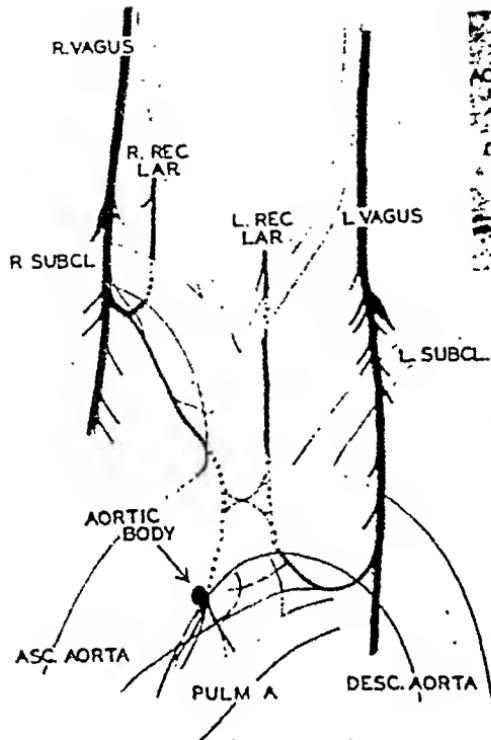


Fig. 2. Localization of the chemoreceptors of the aorta in dog. Right top: microphotography of the aortic body with its aortic branch. Below is seen the effect, in connection with electric stimulation, on the respiration and blood pressure of the afferent branch to the aortic body (J. H. COMROE: Am. J. Physiol. 1939, 127, 176).

By connecting the pump with bags containing various gas-mixtures it was possible to study the effect of these on the action potentials that were elicited by chemical stimuli.

## 2. Localization.

Since in their fundamental work J. F. HEYMANS and C. HEYMANS (1927) showed that impulses stimulating the respiration could be elicited through chemical stimulus on perfusion of the heart and the nearest part of the aorta of dog with the head,

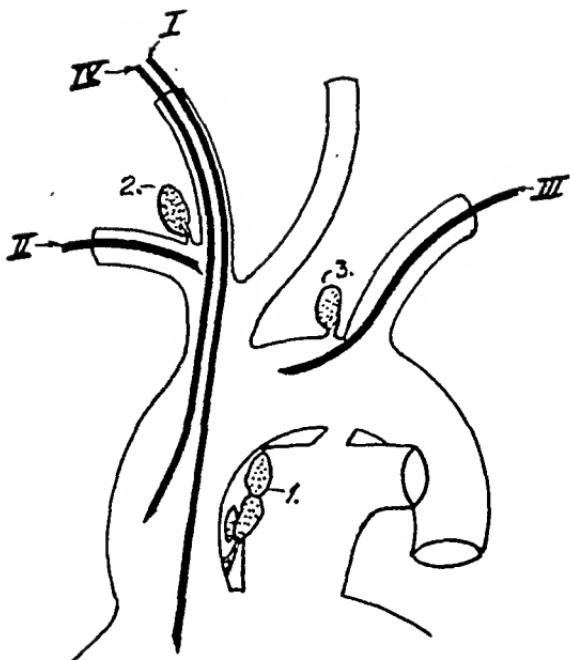


Fig. 3. Schematic drawing of the localization of certain so-called paraganglia (in part after NONIDEZ: Amer. J. Anat., 1935, 57, 259). The figures 2 and 3 refer to the paraganglia described by MURATORI and NONIDEZ; and 1 marks the aortic body. I, II, III and IV mark various catheter positions. For further explanation see text.

apart from the vago-depressors, entirely isolated from the body, no new attempt at a closer study of the exact situation of this chemo-sensitive zone was carried out until COMROE (1939) tried to locate the position more exactly in dogs and cats, taking a physiological line of approach. By means of local application in the aorta of dogs with denervated carotid sinuses of certain stimulants (cyanide, lobeline) through injection via a fine catheter, he was able to get an idea of the spot where the strongest reflex excitation was released. With this procedure, combined with local denervation, to which I shall revert later, he was always able to localize the chemo-sensitive region to aorta ascendens or to the beginning of the aortic arch (Fig. 2). This chemo-sensitive region of the aorta will be referred to in the

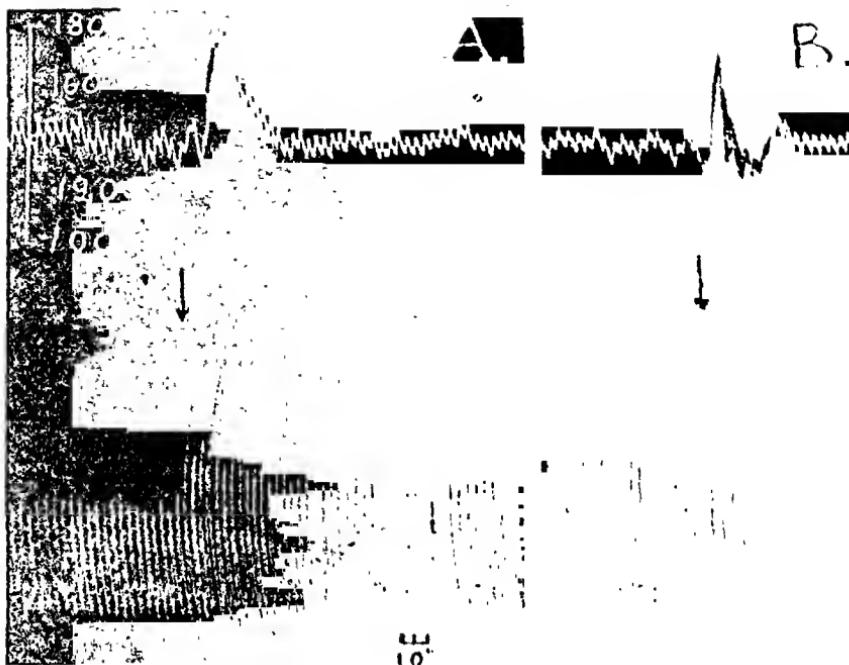


Fig. 4. Cat 2.2 kg. After bilateral sinus denervation. The upper curve blood pressure, lower curve respiration (pneumograph). At the arrows injection through a catheter of 0.3 mg of lobeline hydrochloride. In A the tip of the catheter is placed just inside the aortic valves. In B the catheter tip lies outside the aortic valves in aorta ascendens.

sequel as *the aortic body*, under which name it is most commonly known in the literature.

If the opening of the catheter was placed just inside the aortic valves (Fig. 3, catheter position I) of an animal with denervated carotid sinuses one obtained a prompt increase of respiration on injection of 0.3 mg of lobeline hydrochloride (Fig. 4A). This is in full agreement with COMROE's experiments on cat. With a view to the paraganglia described by MURATORI and NONIDEZ the catheter was introduced through the right or left subclavian artery so that the opening came to lie in the spot from which the vessels proceeded which supplied these formations with blood (Fig. 3, catheter positions II and III). But in these positions no

increase at all in the respiration was obtained on injection of lobeline, even in doses far exceeding that given above. Bearing in mind the possibility that owing to the blood stream in these catheter positions the lobeline did not reach these paraganglia, lobeline was injected in aorta ascendens via the catheter (Fig. 3, catheter position IV) but still without any increase in the respiration (Fig. 4B). One arrived at the same result also when the action potentials were recorded from the depressor nerve. In catheter position I one obtained a strong increase in the chemical impulses on injection of lobeline, but this increase was entirely absent in catheter positions II, III and IV. It would thus appear that from these paraganglia one cannot elicit any reflex effect on the respiration with a chemical stimulus. On the other hand, it is easy to produce such an effect from the paraganglia described by PENITSCHKA (paraganglion aorticum supracardiale) (Table 1 and Fig. 1). Further proof of this opinion will be given below.

### 3. Nerve supply.

Since HEYMANS and HEYMANS showed that the afferent impulses which arose on chemical stimulus of the aortic region in the dog passed in the vago-depressors, this has been verified by a large number of investigators, and it is now generally accepted as a fact. COMROE (1939) found, as was expected, that in dogs with denervated sinuses no stimulating effect could be obtained on the respiration and blood pressure on stimulation with hypoxemia, lobeline or cyanide if the vago-depressors were severed. On the other hand, an effect could be produced with these stimuli if the vagi were severed below the point of entry of the depressor nerves. This shows, as was already known, that the afferent path for the impulses from glomus aorticum is the vagi via the depressors.

On dissection of the depressor nerves in cat it proves that these join the vagus trunk immediately below nervi recurrentes or together with these. But from the depressor nerves there proceed in the majority of animals on both sides one or several branches

which leave the main trunk after approximately half its course from the terminal branching to the point of entry into the vagus. These branches, which are extremely thin, turn off more sharply laterally and join the vagus 1 to 2 cm below the point of entry of the depressor nerve proper. Such thin nerve fibres between the depressor nerve and vagus have been described by PERMAN (1920), but were then localized somewhat higher up. If one records the action potentials from these "accessory" branches from the depressor nerve one gets the same picture as from the main trunk of the nerve, thus the ordinary fast pressor impulses at each systole as well as the slower chemical potentials elicited by e.g. hypoxemia or different drugs (lobeline, piperidine etc.). These "accessory" branches from the depressor nerves may possibly provide the explanation of the fact that in cases of hypoxemia the effect on the respiration after severing the earotid and depressor nerves to some extent remains, and is not entirely lost until also the vagi are severed (JONGBLOED 1936, LAMBERT and GELLHORN 1938). Presumably only the main trunk of the depressor nerves, which is from the technical point of view easier to get at, has been severed, while impulses from the chemosensitive zone of the aorta have been able to pass in the "accessory" branches, which join vagus further down, and have then passed up and thus produced an effect on the respiration.

In accordance with the theory advanced by DE CASTRO (1927), HEYMANS and BOUCKAERT (1933) were able to show that the receptor organs for the chemical stimulus in the sinus region are not identical with or localized to the same spot as the baroreceptors. COMROE (1939), in one experiment on dog with denervated earotid sinuses, was able to destroy mechanically the nerves running in the vicinity of the aortic body, and thus prevent the release of the chemical reflex, while the pressor reflex remained unchanged. This is in full agreement with the experiments carried out by BOUGE and STELLA (1934, 1935), and by ZOTTERMAN (1935). In experiments on the carotid sinus, with recording of the action potentials from Hering's nerve by severing or squashing the nerve fibres from the chemo-sensitive or pressor-sensitive regions, these authors were able to obtain either chemical or pressor impulses.

Taking as the point of departure these earlier investigations, it has been possible, in connection with the recording of the action potentials in the depressor nerve, to make a closer study of the result of a local destruction of the terminal branchings of this nerve in the region around the concave side of the aortic arch. By squeezing with a pair of tweezers in the region between aorta ascendens and the pulmonary artery, thus the site of the aortic body, it was fairly easy to destroy the fibres leading the afferent impulses from the chemo-sensitive region. By subsequent checking of the effect of lobeline or hypoxemia on the chemical action potentials in the depressor nerves it was possible to convince oneself that these were now no longer obtained, but that the pressor impulses remained apparently unchanged. In one case an involuntary exclusion of the chemical impulses from a previously good subject was obtained, when in connection with an intravenous injection of lobeline the cat gave a jerk, so that the lifted depressor nerve, which was tied with a silk thread with a fixed point of attachment, and from which the action potentials were recorded, was over-stretched. After this, no further chemical impulses were obtained, while on the other hand, pressor impulses came as before. But I never succeeded entirely in only destroying those nerve fibres which conducted the pressor impulses, without at the same time more or less destroying the nerve fibres from the aortic body. Since the pressor impulses, as compared with the chemical impulses, are released from such a relatively large region, there are great technical difficulties connected with the destruction of these without the chemical fibres becoming at the same time involved in the process. These experiments, however, support the assumption that the chemoreceptors in the aorta as well as in the carotid sinuses are anatomically distinguishable from the baroceptors.

In view of the fact that in rabbit the main trunks of the depressor nerves run isolated from the vagi, this animal should provide better conditions for investigations of the chemo-sensitive aorta zone. One would in this ease be able to record the action potentials from the nerve where it runs in the neck, without being compelled in the majority of cases, as one is with the cat,

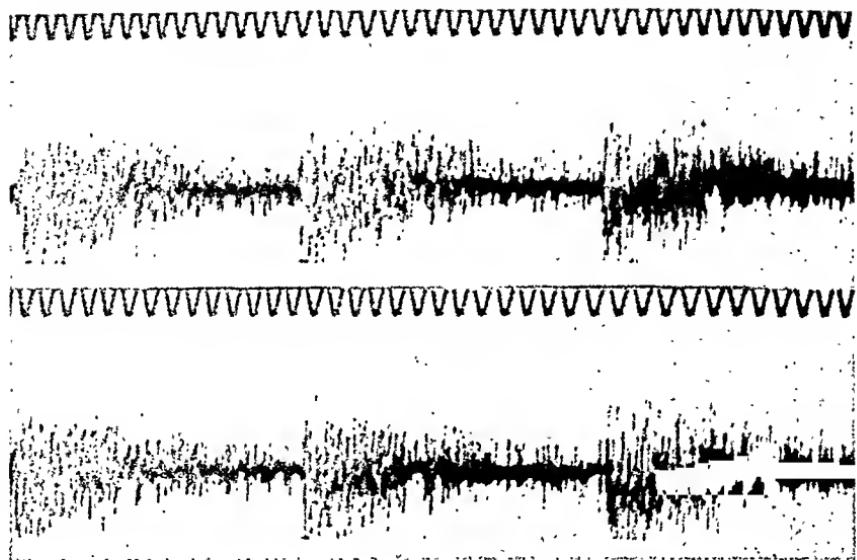


Fig. 5. Rabbit 2.4 kg. Action potentials from the right depressor nerve.  
A. Control. B. After intravenous injection of 1 mg lobeline hydrochloride.  
Time: 1/50 sec.

to make a resection of the uppermost ribs on one side and on this account being forced to administer artificial respiration to the animal throughout the experiment. In exceptional cases, however, one finds that also in cat the depressor nerve runs isolated from vagus for a longer or shorter stretch in the neck instead of, as in most cases joining the vagus already in the thoracic cavity. It now proved, however, that on chemical stimulus of the presumed chemo-sensitive aortic zone in rabbit and with simultaneous recording of the action potentials from the depressor nerve no change at all of the electro-neurogram could be obtained to indicate the existence of chemical impulses (Fig. 5). In order to produce a chemical stimulation recourse was had as usual to lobeline, cyanide or piperidine (intravenously or locally by intracardial injection) and to hypoxemia varying from 12 % to 6.0 % O<sub>2</sub> in N<sub>2</sub>. If these animals were allowed to breathe a gas mixture poor in oxygen (7.3 % O<sub>2</sub> in N<sub>2</sub>) during the recording of the respiration with a Marey pneumograph, one obtained the

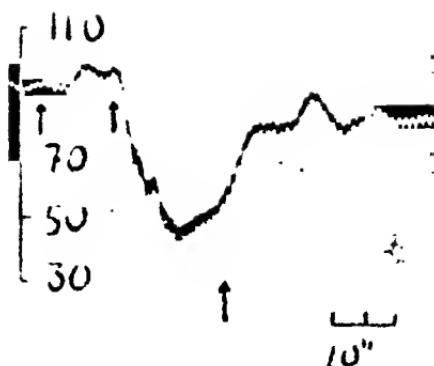


Fig. 6. The same rabbit as in Fig. 5. Registration of blood pressure. The first arrow indicates the moment when the electro-neurogram A in Fig. 5 is taken. The second arrow indicates the point of time for the injection of 1 mg lobeline hydrochloride intravenously. The third arrow marks the point of time when the electro-neurogram B is taken.

ordinary increase in the respiration. If after this one severed the two depressor nerves and repeated the experiment one obtained no measurable change in the increase in the respiration. On intravenous injection of 1 mg of lobeline hydrochloride the case was the same, namely, that the severing of the depressor nerves did not reduce the stimulating effect on the respiration. In rabbit, the intravenous injection of lobeline in doses of 0.5—1 mg always caused a reduction of the blood pressure (Fig. 6) instead of, as with the cat, causing a rise in the blood pressure.

Not all the fibres of the depressor nerves in the rabbit run in complete isolation from vagus, they also sometimes occur to some extent together with the latter. This is also observed if one splits up the vagus in thin fascicles and records the action potentials from one of these. In this case one can sometimes obtain a fascicle containing depressor fibres, which may be recognized as conducting the fast impulses that are elicited by each pulse wave. It is therefore possible that the afferent, chemical impulses might travel in the fibres that were enclosed in the vagus trunk or in vagus and not in the isolated depressor nerve. Any attempt to lead

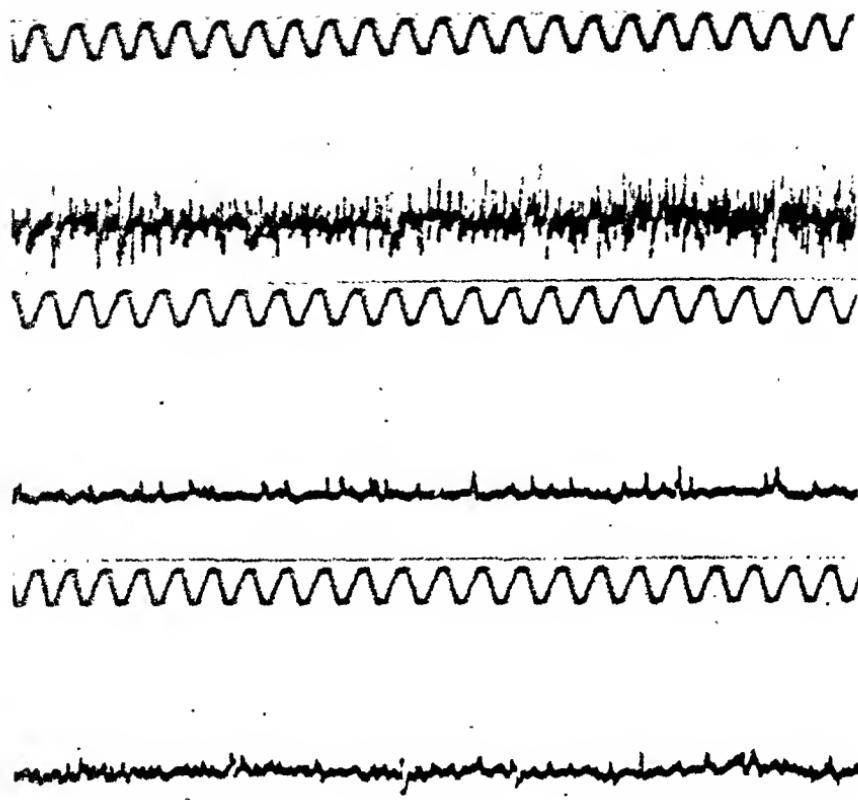


Fig. 7. Rabbit 2.0 kg. Recording of the action potentials from a thin fascicle of the right vagus. A. Control. B. After spraying of trachea and right bronchial system with 2 % pantocaine solution. C. After intravenous injection of 0.75 mg of lobeline hydrochloride. Time: 1/50 sec.

off these specific impulses from the vagus after chemical stimulation of the aortic body presents, however, as already stated, great difficulties on account of the very strong volley of impulses that is released with each breath. GERNANDT and ZOTTERMAN (1945) met with these difficulties in connection with their attempts to find afferent vagus fibres that responded to increased pressure in the central veins (the Bainbridge reflex). By means of various procedures they attempted to eliminate the afferent fibres of the lungs. The best result was obtained by introducing

blood was used for estimation of histamine in plasma. The skin samples weighed 25—75 mg. The results, summarized in table 4, agree with those of table 3.

TABLE 4.

Rat nr	1			2			3		
Date	19.4	3.5	30.5	23.4	2.5	17.5	19.4	2.5	24.5
Plasma Hi, γ/l	140	145	150	250	225	225	150	165	150
Skin Hi, γ/l	24.8	27.9	22.3	30.0	32.0	35.5	19.5	16.5	20.5
Rat nr	4			5			6		
Date	18.4	17.5	20.4	2.5	19.4	17.5	17.4	3.5	1.6
Plasma Hi, γ/l	110	100	190	220	315	300	145	145	165
Skin Hi, γ/l	10.9	15.0	—	33.7	37.5	32.8	28.2	31.9	27.0

c. *Experiments on dogs.* The histamine content of plasma was determined in two dogs. In an ear vein of a nonanaesthetized dog an incision was made from which about 20 ml of blood was collected in a centrifuging tube, containing heparine. The sample was treated as in the previous experiments. Table 5 demonstrates the constancy of the plasma values.

TABLE 5.

Dog nr	1				2			
Date	20.2	2.3	15.3	7.4	27.2	6.3	8.3	18.5
Plasma Hi, γ/l	40	35	40	40	60	60	55	55

d. *Experiments on rabbits.* 10—15 ml of blood was collected from an ear vein of the nonanaesthetized animal. From table 6 it is obvious that the histamine concentrations of the plasma samples show very great variations while the histamine content of blood remains fairly constant.

In this respect the rabbit obviously differs from the other

lobeline the same view has been arrived at in these investigations on recording the respiration and the action potentials from the depressor nerve. A catheter was introduced in the usual way through the common carotid artery into the left ventricle of cats with denervated carotids. If the opening of the catheter was placed inside the valves of the aorta and lobeline was injected one obtained a prompt effect on the respiration and blood pressure. If the catheter was placed in such a way that the opening lay distally to the valves, this effect on the respiration did not appear (Fig. 4).

On the injection of cyanide or lobeline in the right ventricle COMROE showed that there was always a longer interval between the injection and the appearance of an effect on respiration and blood pressure than after the same injection in the left ventricle. This would support the assumption that the chemoreceptors in the cat, as also in the dog, do not obtain blood from the pulmonary artery.

#### IV. The effect of hypoxemia and hypercapnia on the chemoreceptors in the aortic body

That chemical stimuli (hypoxemia, carbon dioxide, substances having an effect like that of nicotine) exercise a stimulating effect on the respiration (HEYMANS, BOUCKAERT and DAUTREBANDE 1930, 1931) and the circulation (HEYMANS, BOUCKAERT and DAUTREBANDE 1930, 1931) and the circulation of the blood (HEYMANS, BOUCKAERT, EULER and DAUTREBANDE 1932), through a reflex stimulus of centres released from the chemoreceptors in the carotid bodies and in a lesser degree from the aorta region, has, since the fundamental investigations of Heymans and his co-workers, been confirmed by a large number of researchers. The results of experiments upon non-anaesthetized, anaesthetized or decerebrated rabbits, cats and dogs have on the whole been in agreement. The authors have employed in part the simple denervation procedure and in part the crossed circulation method introduced by HEYMANS and HEYMANS.

The effect produced by hypoxemia on the chemoreceptors has also been studied on the action potentials, which have been recorded from Hering's nerve. After BRONK (1931) had shown in experiments on rabbit how each systole released a wave of big, fast impulses, whereas during diastole there was a wave of big, slight activity which increased on high blood pressure or on asphyxia, attention was turned in a couple of subsequent or on by BRONK and STELLA (1932 a+b) especially to the occurrence of impulses of a more continuous type between the big volleys during the systole. They advanced the assumption that these small, continuous impulses were due to chemical factors, in agreement with the observations made earlier by HEYMANS, BOUCKAERT and DAUTREBANDE concerning the effect of a change in the blood gases on the reflexes from the carotid sinus. The following year also HEYMANS and RIJLANT (1933) observed the occurrence of

these two different types of action potentials on recording from the sinus nerve. The big ones were due to pressure variations in the sinus, the small ones were caused by the changes in chemical composition of the blood. The latter kind of impulses, which were of the continuous type, proved to have a considerably lower frequency (about 20—40 per second) than those which were released by the endosinusal pressure variation, which had a frequency of about 100 per second. The small impulses remained even if the carotid artery was clamped below the sinus region, so that the impulses produced by the pulse wave were eliminated. The assumption of these authors, that these small action potentials are caused by the chemical stimulus of the blood, was confirmed by later investigations carried out by BOUCÉ and STELLA (1934, 1935) and ZOTTERMAN (1935). Taking as their point of departure the already mentioned observations made by HEYMANS, BOUCKAERT and DAUTREBANDE, these authors succeeded in mechanically excluding the baroceptors, and were thus able to get a clearer picture, especially of the chemical action potentials. ZOTTERMAN showed that the amplitude of these small potentials were only 10—20 % of the largest spike potentials which were elicited by stretch and that there was the same relation between the amplitude of the taste potentials and the largest touch potentials recorded from the glossopharyngeal nerve running to the tongue. BOUCÉ and STELLA found that on a stronger stimulation of the chemoreceptors the chemical potentials were considerably greater. This is presumably due to a summation effect. These authors and Zotterman were able to show that the number of chemical impulses increased considerably in connection with hypoxemia or asphyxia.

The assumption of HEYMANS and his co-workers that the occurrence of a chemical sensibility in the sinus and aorta regions was bound to specific receptors distinguished from baroceptors received strong support in DE CASTRO's (1926, 1927) morphological investigations on the receptors in the sinus region. This view has since been confirmed by investigations carried out by SCHMIDT (1932), HEYMANS and BOUCKAERT (1933), GOLLWITZER-MEIER (1934), BRONK and STELLA (1934) and others. The latter

showed that the action potentials deriving from the baroceptors were insensitive to changes in the oxygen-content of the blood on perfusion of the isolated sinus with arterial blood or blood poor in oxygen. They arrived at the same result also on variation of the carbon dioxide tension of the blood.

The chemoreceptors are normally in a certain state of activity. SAAMAN and STELLA (1935) made a closer investigation of the chemical impulses in the sinus nerve, finding in this connection that these occurred also on respiration under ordinary circumstances. EULER, LILJESTRAND and ZOTTERMAN (1939), on recording the action potentials from the sinus nerve of the cat, carried out a quantitative investigation of the relation between the impulse frequency in the chemical fibres and the oxygen tension of the blood using constant over-ventilation with gas mixtures poor in oxygen. Impulses, obviously elicited by the hypoxemia, appeared as soon as the oxygen tension had sunk only a few per cent. By determining the oxygen tension according to van Slyke's method they found that their animals, which were narcotized with chloralose, generally had a saturation of about 90 %, and from this it emerges that the small impulses, which appear on the spontaneous respiration of air, are at least in part due to hypoxemia. One must also bear in mind, as the authors pointed out, that the content of hemoglobin, the blood pressure and the rate of circulation also play a certain rôle. If the animal was allowed instead to breathe pure oxygen, the frequency of these small impulses was reduced. This is in agreement with ZOTTERMAN (1935), who showed that the small chemical impulses could be eliminated by blowing oxygen into the trachea. EULER and ZOTTERMAN (1942) showed that it was also possible to record action potentials, caused by chemical stimuli from the sinus nerve of the dog.

### 1. Hypoxemia.

On recording the action potentials from the depressor nerves one finds that, apart from the fast impulses produced by every systole, there is a continuous stream of small impulses. These latter must, in the light of earlier investigations, be considered

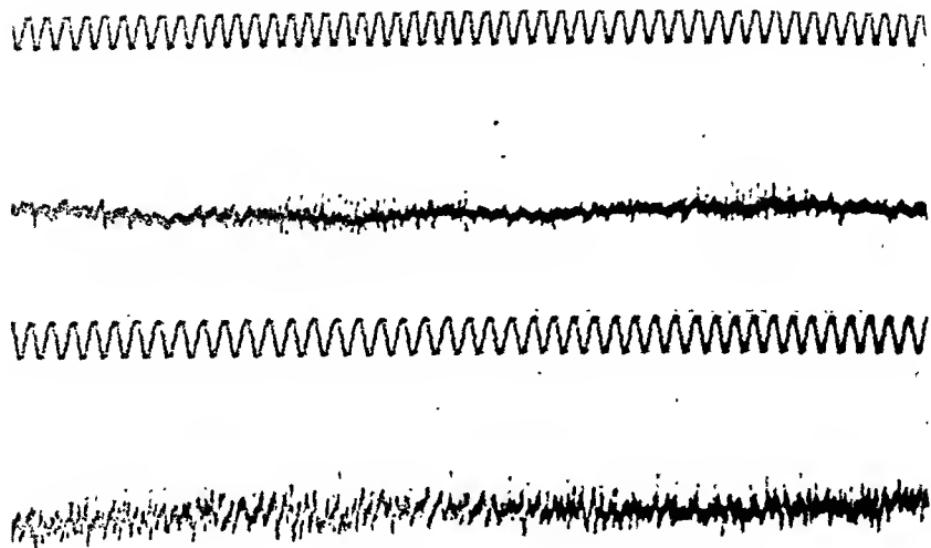


Fig. 8. Cat 3.0 kg. Artificial respiration, constant over-ventilation with air. Recording of the action potentials from the right depressor nerve. A. Control, BP 110 mm. Hg. B. 6.8 % oxygen in nitrogen, BP 120 mm. Hg. Time: 1/50 sec.

to be caused by the chemical stimulus of the blood, or in other words to be elicited by hypoxemia, carbon dioxide or both factors. By increasing the artificial ventilation, so that the carbon dioxide tension was kept under the threshold value for stimulation and the oxygen tension of the blood was increased, a clear reduction of the small, chemical impulses was obtained.

After this, and with the same ventilation, the effect of gas mixtures poor in oxygen, varying between 5.6 to 12 % oxygen in nitrogen, was tested. Fig. 8 shows the effect on the chemical action potentials of ventilating the lungs with 6.8 % O<sub>2</sub> in N<sub>2</sub>. One sees here the typical increase of the action potentials produced by the chemical stimulus, which is in full agreement with similar experiments on Hering's nerve.

Fig. 9 shows the effect on the chemical action potentials from the depressor nerve on ventilation with pure oxygen. This caused an almost complete disappearance of all the small impulses.

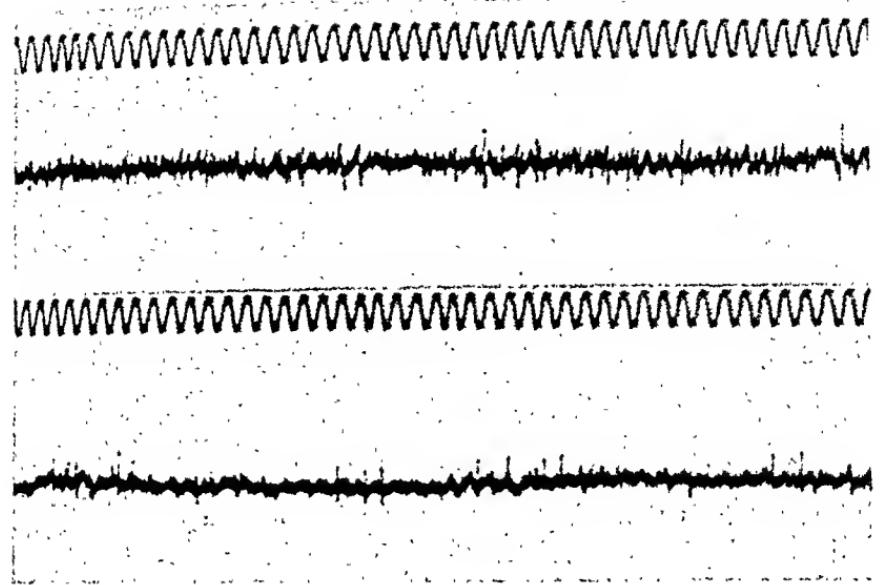


Fig. 9. Cat 3.0 kg. Artificial respiration air. Recording of the action potentials from the right depressor nerve. A. Control, BP 145 mm. Hg. B. 100 % oxygen, BP 140 mm. Hg. Time: 1/50 sec.

While the effect on respiration and blood pressure produced by the hypoxemia goes almost entirely via the chemoreceptors, the case as regards carbon dioxide is complicated by the fact that a part of the effect is direct on the centre. HEYMANS, BOUCKAERT and DAUTREBANDE (1930) were able in perfusion experiments on dog to show from the sinus region, which was isolated in respect of the circulation, that a rise in the carbon dioxide tension caused a reflex increase in the respiration over the chemoreceptors. The carbon dioxide still had, however, a stimulating effect on the respiration after severance of the buffer nerves, so a direct effect upon the respiratory centre had also to be assumed. Special experiments, however, led them to adopt the view that the sensitiveness to carbon dioxide of the reflexogenic zones was greater than that of the respiratory centre. As a result of further study, HEYMANS and BOUCKAERT (1939) found that the effect over the chemoreceptors is quicker in starting than that over the

centre, whose reaction is slower but longer lasting. GOLLWITZER-MEIER and LERCHE (1940) observed also that carbon dioxide was active over the chemoreceptors but they were on the other hand of the opinion that the threshold value of the latter was higher than that of the respiratory centre. The chemoreceptors therefore had only an auxiliary function, which set in only when higher concentrations of carbon dioxide had been reached, but within physiological limits.

Although it is difficult to draw a comparison here, as an isolated elimination of either organ can only be realized as far as the sinus and the aortic bodies are concerned, it nevertheless emerges beyond doubt from experiments by EULER and LILJESTRAND (1940) that the respiration was reduced after denervation of the reflexogenic zones in the sinuses also after it had been possible to exclude hypoxæmia. The view advanced by OWEN and GESELL 1931, SCHMIDT 1932, GEMMILL and REEVES 1933, WRIGHT 1934, 1937, STELLA 1935, GESELL and MOYER 1937 and SMYTH 1937, that carbon dioxide has no effect over the chemoreceptors, is probably in some way connected with unfavourable experimental conditions.

The real question here is whether under physiological conditions carbon dioxide exercises on the respiratory centre any stimulating effect whatever that is reflexly elicited from the sinus and aorta regions. SCHMIDT and COMROE (1940) have strongly denied any such effect under physiological conditions.

SAAMAN and STELLA (1935), recording the impulses from Hering's nerve, found that the threshold value for stimulation of the chemoreceptors for carbon dioxide lay at a carbon dioxide tension of about 33—35 mm Hg in the arterial blood. This agrees well with the values obtained in the experiments by EULER, LILJESTRAND and ZOTTERMAN (1939). They found also that such carbon dioxide concentrations in the gas mixture supplied on artificial ventilation which led to alveolar carbon dioxide values entirely within the normal limits brought about a marked increase in the impulse frequency. On increase of the carbon dioxide content the impulse frequency rose in an approximatively linear relation. They therefore considered that carbon dioxide also had

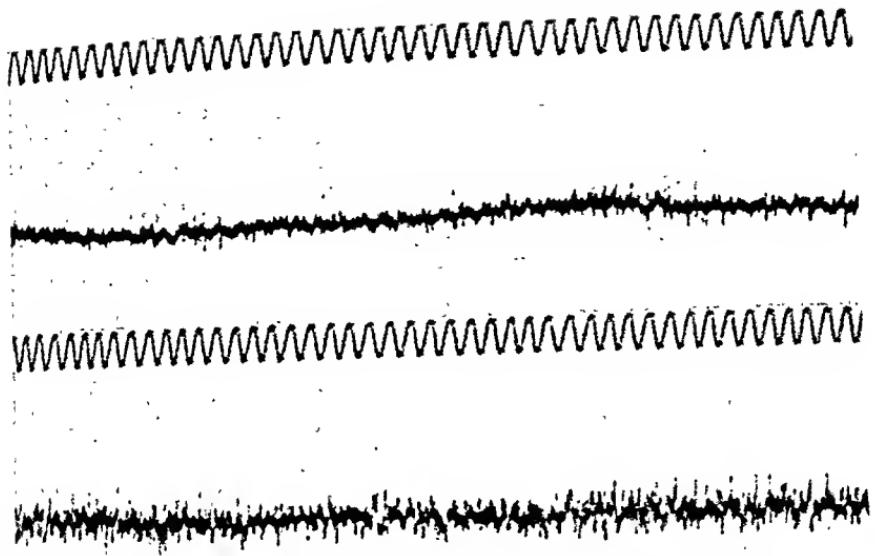


Fig. 10. Cat 3.0 kg. Artificial respiration, constant over-ventilation with air. Recording of the action potentials from the right depressor nerve. A. Control, BP 110 mm. Hg. B. 6.3 % carbon dioxide in oxygen, BP 115 mm. Hg. Time: 1/50 sec.

a stimulating effect on the centre that was reflexogenically elicited from the carotid sinuses.

It is probably now beyond doubt that under normal conditions carbon dioxide exercises a reflex stimulating effect over the chemoreceptors in the sinus and aorta regions, but that in the absence of this the central effect on the respiratory centre is still in a position to regulate the ventilation, although at a higher threshold level, as is shown, moreover, by the fact that apnoea may sometimes appear on denervation.

## 2. Hypercapnia.

In order to be able to study the effect of carbon dioxide on the aortic body, the animal was kept in a slight over-ventilation with an oxygen mixture in which carbon dioxide entered in different

concentrations. This was to ensure that the animal should be fully saturated with oxygen, so that all action potentials that were caused by hypoxemia might be eliminated. Fig. 10 shows the effect of 6.3 % CO<sub>2</sub> in O<sub>2</sub>. One sees the increase of the chemical impulses, which must be considered to be produced by the increased carbon dioxide tension in the blood.

This is in full agreement with earlier experiments on the afferent impulses in Hering's nerve under similar conditions.

## V. The effect of various drugs on the chemoreceptors in the aortic body

The following investigations show the stimulating effect of certain drugs causing an increase in the respiration and the blood pressure on the chemoreceptors in the aortic body, with simultaneous recording of the action potentials from the depressor nerve. In this section the main interest has been devoted to the chemical action potentials. In a later connection the blood pressure reactions will also be partly studied.

### 1. Lobeline.

The stimulating effect of lobeline on the respiration has long been known. It was at first assumed that the strongly stimulating effect of small doses was localized to the respiratory centre. Large doses caused paralysis of the respiration. In investigations by HEYMANS, BOUCKAERT and DAUTREBANDE (1931), however, the question appeared in a new light, as in experiments on dogs these authors were able to show that the injection of lobeline in small doses (0.01—0.1 mg) into the common carotid artery caused a marked increase in the respiration, which did not result, however, if the carotid sinuses had been denervated. Also with intravenous injection of lobeline in doses of 0.5—1 mg this effect on the respiration was obtained; but after denervation of both the carotid sinuses and severance of the vago-depressors on both sides the effect was almost entirely absent. This led the authors to assume that the stimulating effect of lobeline on the respiration is mainly reflexly conditioned over the carotid sinuses. CLEMENTI (1928) and CRIMI (1933) applied lobeline direct to the bottom of the fourth ventricle and obtained, here too, a stimulation of

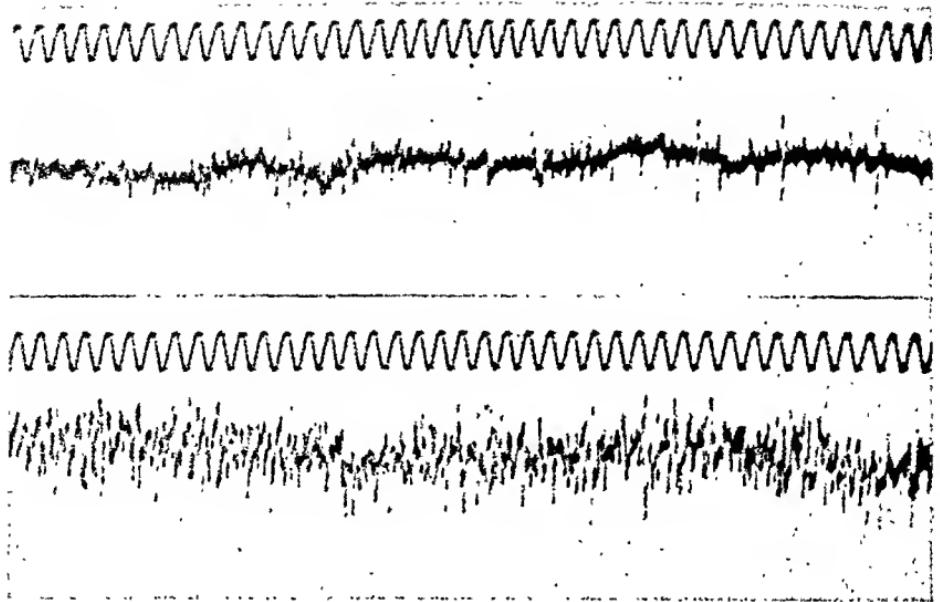


Fig. 11. Cat 3.2 kg. Artificial respiration air. Recording of the action potentials from the right depressor nerve. A. Control, BP 165 mm. Hg. B. After intravenous injection of 1 mg lobeline hydrochloride, BP 190 mm. Hg. Time: 1/50 sec.

the respiration. It would thus appear that under certain circumstances also the respiratory centre can be stimulated by lobeline. GOLLWITZER-MEIER (1934) was also able to constate that lobeline takes effect of the sinus region, but scarcely, on the other hand, on the respiratory centre. EULER, LILJESTRAND and ZOTTERMAN (1939), in connection with the registration of impulses from Hering's nerve, were able to show that lobeline causes a much stronger increase of the chemical impulses than hypoxemia is ever able to bring about.

In my experiments on the stimulating effect of lobeline on the chemoreceptors in the aortic body the lobeline has been given in part intravenously and in part intracardially (catheter position I, Fig. 3, p. 23). Fig. 11 shows the effect of 1 mg of lobeline hydrochloride intravenously upon the inflow of impulses in the right depressor nerve. One sees how the lobeline causes a very marked

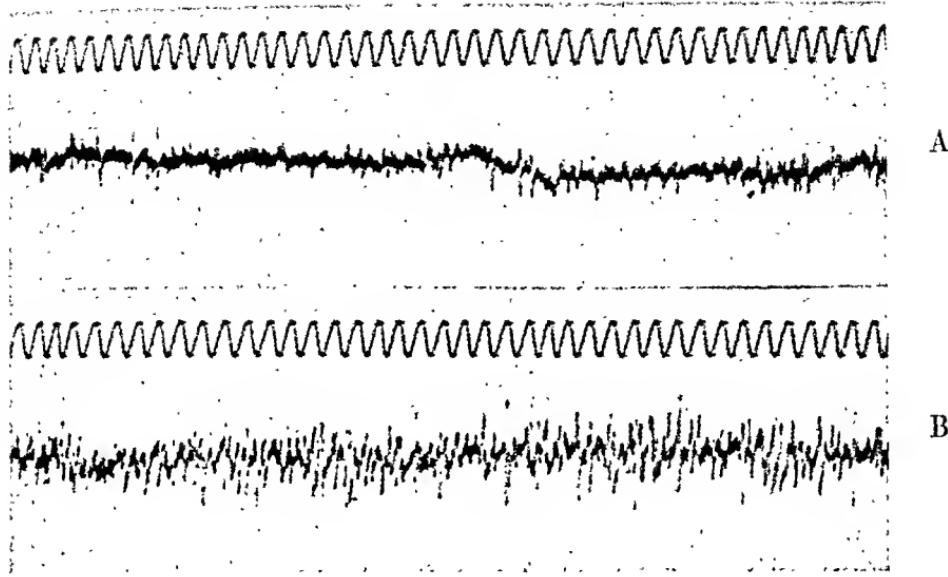


Fig. 12. Cat 3.2 kg. Artificial respiration air. Recording of the action potentials from the right depressor nerve. A. Control, BP 160 mm. Hg. B. After intravenous injection of 1 mg piperidine hydrochlorid, BP 190 mm. Hg. Time: 1/50 sec.

increase of the chemical impulses, which gradually disappeared in the course of some minutes. Also the fast and larger spike potentials increased, but this was due to the rise in the arterial blood pressure.

## 2. Piperidine.

EULER (1945) showed that piperidine is normally excreted with the urine, and that this excretion increases considerably on muscular exertion. Its typical nicotine-like pressor effect made it probable that it could stimulate the chemoceptive fibres in the carotid bodies. GERNANDT and ZOTTERMAN (1945) were able to show that piperidine has an effect identical with that of lobeline on the chemoreceptors in the carotid sinus.

Fig. 12 shows the effect of intravenous injection of 1 mg

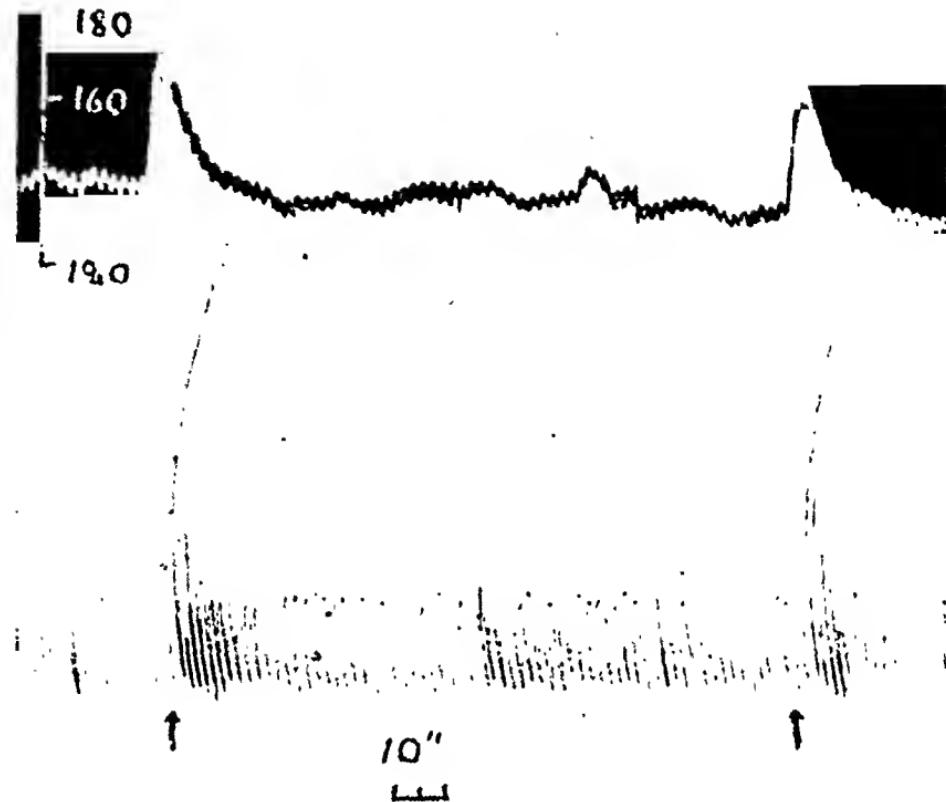


Fig. 13. Cat 3.8 kg. After bilateral sinus denervation. Upper curve blood pressure, lower curve respiration recorded with a pneumograph. At first arrow injection via a catheter with the tip inside the aortic valves of 0.3 mg lobeline hydrochloride; at second arrow injection of 0.3 mg piperidine hydrochloride.

piperidine hydrochloride on the chemoreceptors in the aortic body by recording the impulses from the depressor nerve. A comparison with Fig. 11 shows this effect of piperidine to be identical with that released by lobeline.

Fig. 13 shows the effect, on local, intracardial injection, of 0.3 mg lobeline hydrochloride and 0.3 mg piperidine hydrochloride on the respiration and blood pressure of an animal with severed sinus nerves.

### 3. Cyanide.

GEPPERT (1889) pointed out that the toxic effect of the cyanides was due to the fact that they caused an internal asphyxia. Consequently, owing to a blockade of certain oxidation enzymes, the cyanides have the same effect on the respiration as has hypoxemia, and various researchers have also been able to show this. HEYMANS, BOUCKAERT and DAUTREBANDE (1931) showed that the stimulating effect of cyanide on the respiration was caused mainly by a reflex effect from the carotid sinus, and that the sinus was much more sensitive to this stimulus than was the respiratory centre, which was practically insensitive. The same thing also applies to the effect on the blood pressure. OWEN and GESELL (1931), however, found that cyanides could produce a certain increase in the respiration even after complete bilateral sinus denervation. HEYMANS, BOUCKAERT and RECNIERS (1933) also arrived at the same result. WRIGHT (1935) observed that after cyanides there appeared two phases in the increase in respiration. The first was reflex-conditioned and was presumably caused by the hypoxemia occurring in the chemoreceptors; the second stimulation of the respiration, which lasted longer, was not changed by denervation of the sinuses. The latter was considered by EULER and LILJESTRAND (1937) to be due to the formation of acid metabolites, which arose in consequence of the reduced oxidation in the whole body and which caused a stimulation of the respiratory centre and also of the chemoreceptors if these were intact. This view is also supported by an investigation by GESELL, KREUGER, GORHAM and ROSENTHAL (1930), who found that intravenous injection of cyanide was followed by a rapid increase of the lactic acid in the blood as well as by a rise in the hydrogen-ion concentration of the arterial blood.

On intravenous injection of Ringer's solution containing 0.02% of potassium cyanide, with simultaneous registration of the action potentials in the depressor nerve one obtains a clear increase of the chemical impulses. The electro-neurogram in Fig. 14B was taken 1½ minutes after the commencement of the infusion. The animal had then received 3.2 ml of potassium cyanide solution.

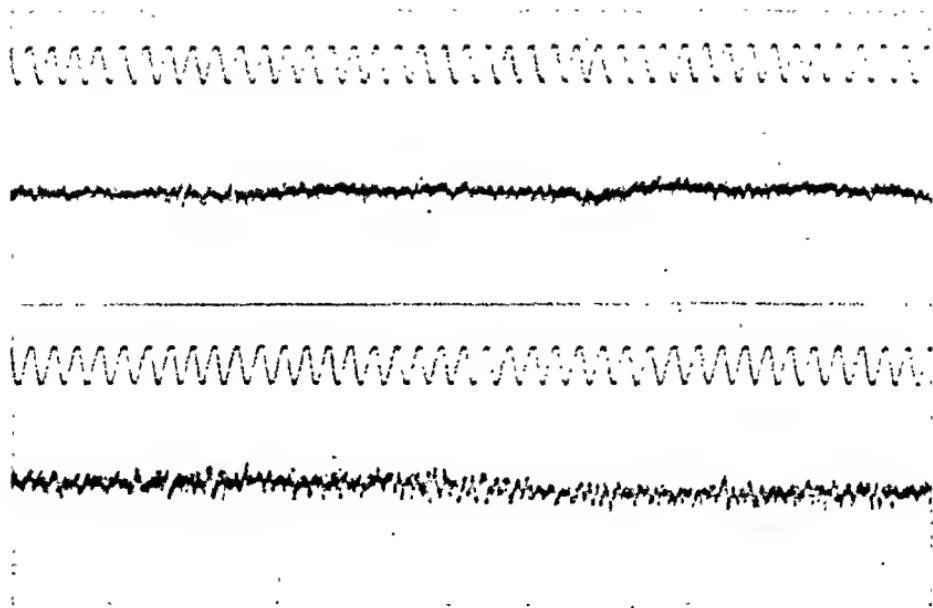


Fig. 14. Cat 2.9 kg. Artificial respiration air. The action potentials from the right depressor nerve. A. Control. B. During infusion of a weak cyanide solution. Time: 1/50 sec.

This is in agreement with SAAMAN and STELLA (1935), who recorded action potentials from Hering's nerve and found an increase of the chemical impulses after cyanide. EULER, LILJESTRAND and ZOTTERMAN (1939) found that if in connection with the continuous supply of potassium cyanide solution, with simultaneous recording of the impulses from the sinus nerve, they administered artificial respiration with pure oxygen instead of air, the chemical impulses caused by the cyanide were reduced very considerably or disappeared altogether. This is in full agreement with the close resemblance between the effect of cyanide and that of hypoxemia.

#### 4. Acetylcholine.

Also this substance has a stimulating effect on the respiration over the chemoreceptors in the sinuses (HEYMANS and HANDOVSKY 1935, HEYMANS, BOUCKAERT, FABER and HSU 1936, SCHWEITZER and

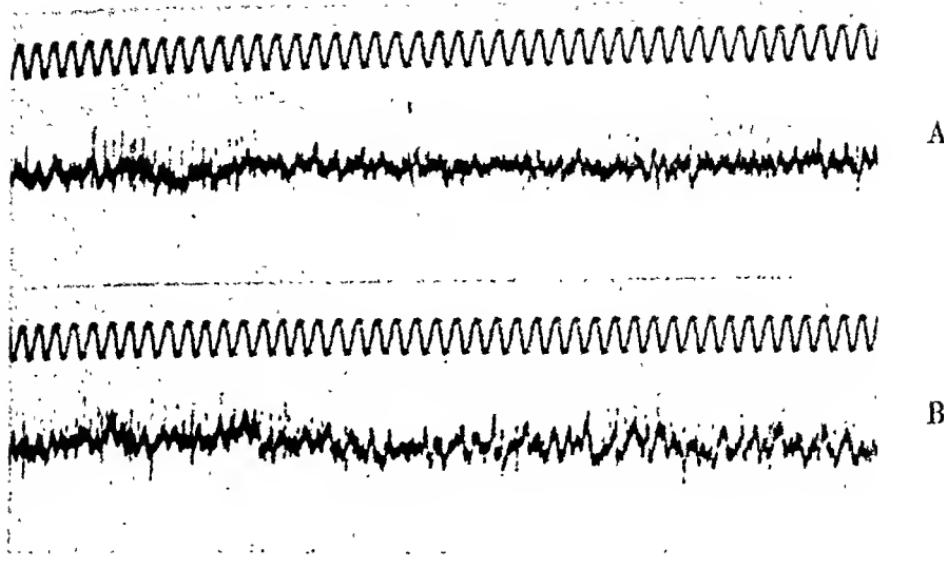


Fig. 15. Cat 2.4 kg. Artificial respiration air. Action potentials from the right depressor nerve. A. Control, BP 155 mm. Hg. B. After injection via a catheter with the tip inside the aortic valves of 10 µg acetylcholine, BP 120 mm. Hg.

WRIGHT 1938, WINDER 1938, EULER 1938, COMROE and SCHMIDT 1938). EULER, LILJESTRAND and ZOTTERMAN (1941), in connection with the intrasinusal supply of acetylcholine, showed how the chemical impulses increased on recording the action potentials from Hering's nerve.

Fig. 15 shows the effect of 10 µg acetylcholine on local, intra-cardial injection. One sees an undoubtedly increase of the typical chemical impulses.

## VI. Selective elimination of the chemoreceptors in the carotid and aortic bodies

In some previous experiments ZOTTERMAN and I observed that strong acids applied intrasinusally could completely extinguish the chemical impulses as was confirmed with recordings from Hering's nerve, while the pressor impulses remained apparently unchanged. EULER, LILJESTRAND and ZOTTERMAN (1939), recording the impulses from the sinus nerve of the cat, showed that after intravenous injection of 0.5 to 2 ml of an 0.5 N ammonia solution all the small impulses disappeared, while the big pressor impulses still appeared as before. If, immediately after this, the animals were allowed to breathe oxygen-poor or carbon dioxide-rich gas mixtures, no increase of the chemical impulses whatever was obtained. Also the ordinary increase in the flow of impulses due to stimulation of the chemoreceptors on infusion of a weak cyanide solution failed to appear after the injection of ammoniac. This is in full agreement with the view that cyanides give rise to hypoxemia. This failure of the stimulating effect was, however, of extremely short duration. After the lapse of a minute or so the chemical impulses returned as before.

Lobeline, nicotine (HEYMANS and co-workers), acetylcholine (HEYMANS and HANDOVSKY 1935) and potassium ions (EULER 1938) have, as has been mentioned, a strongly stimulating effect on the respiration and the blood pressure over the chemoreceptors. They constitute a pharmacodynamically homogeneous group, in so far as all are typical stimulating substances on the transference of stimulus from preganglionic to postganglionic fibres in sympathetic ganglia (synaptotropic substances). It was not possible to inhibit their effect with ammonia. EULER, LILJESTRAND and ZOTTERMAN therefore assumed that these subst-

ances, which evidently do not act in the same way as hypoxenia and carbon dioxide, have their point of attack on some synaptic formation in the carotid bodies. The morphological basis seems to exist, inasmuch as it has been possible to demonstrate the presence of cells in the carotid body partaking of the nature of ganglion cells (DE CASTRO; MEIJLING 1938). If there is a synapse here, higher concentrations of nicotine, according to LANGLEY, would paralyse this. It also proved, in the experiments carried out by EULER et al. that in larger doses both lobeline and nicotine were able entirely to inhibit all respiratory reflexes from the sinuses. The chemical potentials disappeared entirely, while the pressor potentials still came. There can thus in this case not have been any paralysing effect upon the actual nerve fibres, but the observed effect must, in the light of what is so far known, be located to some peripheral synapse or analogous formation. Larger doses of lobeline (1.5 mg) in the carotid artery caused a complete disappearance of all chemical action potentials, but also a reduction of the pressor potentials arose. The same was the case with potassium chloride in larger doses, which had a paralysing effect upon both the chemoreceptors and the baro-receptors (EULER 1938). Curare also had a similar effect (EULER 1940) in this respect, inasmuch as there was an almost complete paralysis of the small impulses but not of the big ones after an intrasinusal injection of 0.25 ml of a 0.2 % curarine solution. On the strength of these and other experiments EULER, LILJESTRAND and ZOTTERMAN therefore came to the conclusion that hypoxemia, carbon dioxide and cyanide take effect on the chemo-receptors in the carotid body, while nicotine, lobeline, acetylcholine and potassium ions exercise their effect somewhat more centrally upon some synapse or analogous formation. In addition to this, ZOTTERMAN (1944) advances the suggestion that the specific action of the lobeline may be located to the junction between the afferent fibres and the chemo-sensitive cells of the carotid body, thus affecting the excitatory transmission between receptor and nerve fibre.

## 1. Technique and procedure.

Experiments have been performed upon cats which were anaesthetized with 0.05—0.07 g of chloralose per kilogram of body-weight. In order to obtain as much space as possible for the electrodes on recording from the sinus nerve, the sinus region was exposed by severing of *musculus sternocleidomastoidens* and by removing the large lymph gland that covers the sinus region. The glossopharyngeal nerve is easily found where it passes along the bulla tympani. After resection of the lateral part of *musculus digastricus* the glossopharyngeal nerve was severed as centrally as possible. By gripping the central end of this nerve it is now possible to dissect out the branch that runs to the carotid sinus. In order, as usual, to obtain as high a signal-to-noise ratio as possible, the sheath of the nerve was drawn off, care being taken to see that the nerve was not exposed to any undue stretching or squeezing. The depressor nerve was dissected out in the way already described.

Into the central part of the lingual artery, which proceeds from the external earotid artery a centimeter or so cranially to the carotid sinus, and which runs along the hypoglossal nerve, a cannula was introduced. After clamping the external earotid artery above the point of departure of the lingual artery on the occasion when the injection was to be made, this cannula enabled a direct intrasinusal injection. A catheter was introduced in the usual way through the common carotid artery and aorta, so that the tip came to lie just inside the aortic valves.

The arterial blood pressure was recorded from the femoral artery by means of an Hg-manometer. Intravenous injections were administered through a cannula in the femoral vein.

## 2. Carotid body.

If on an intrasinusal injection of some few µg of lobeline hydrochloride one obtained the ordinary, tremendously strong increase of the chemical impulses, one might be sure that the chemoreceptors functioned. Lobeline in doses varying between

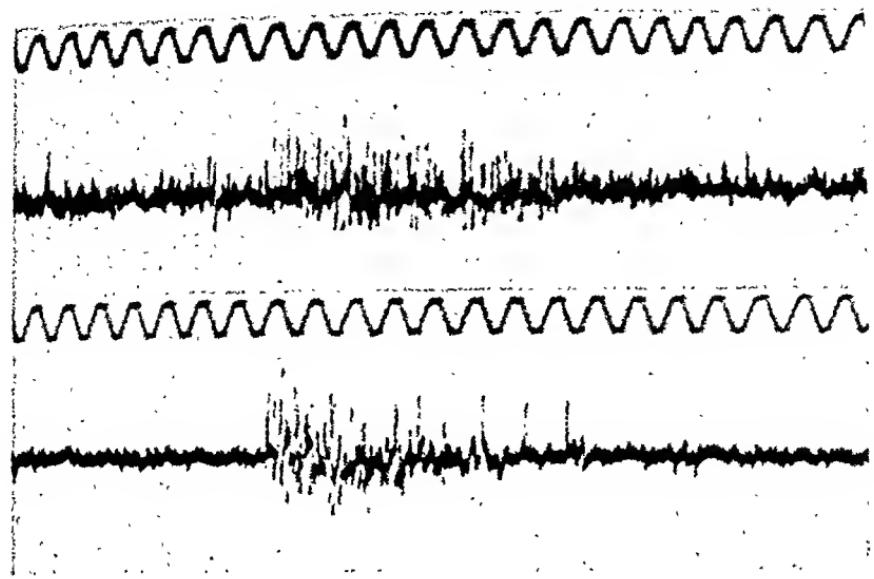


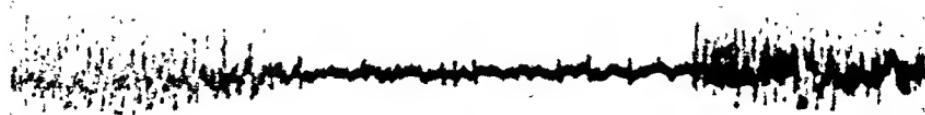
Fig. 16. Cat 3.2 kg. Spontaneous respiration of air. Recording from the sinus nerve. A. Control, BP 130 mm Hg. B. After intrasinusal injection of 0.5 mg urethane in 0.20 ml Ringer's solution, BP 130 mm Hg.

25—50 µg intrasinusally caused, as is known, a temporary reduction or complete extinction of the chemical impulses, while doses of about 0.25 mg generally caused a lasting extinction of both the chemical impulses and the pressor impulses.

On intravenous injection of urethane to narcotize animals, one may observe in connection with a momentary increase in the rate of injection a temporary reduction or standstill of the respiration. In investigation of the effect of urethane on intrasinusal injection it proved that this substance, in doses of about 0.25—0.5 mg, caused for a minute or so a disappearance of the chemical impulses (Fig. 16). This, as well as, without doubt, also a central effect, may provide the explanation of the effect on the respiration that appears in connection with a more rapid injection of urethane.

With 0.5 N acetic acid it was also possible to obtain an extinction of the chemical impulses. If one injected 0.10—0.15 ml of this acid intrasinusally one obtained in the majority of cases an inhibition of the chemical impulses that proved permanent, or

A



B



C



Fig. 17 a. Cat 2.7 kg. Artificial respiration. The action potentials recorded from the sinus nerve. A. Slight over-ventilation with air. Bloodpressure 120 mm Hg. B. Same ventilation but with 10.5 % oxygen in nitrogen. BP 130 mm Hg. C. After intravenous injection of 1 mg lobeline hydrochloride. BP 160 mm Hg. Time: 1/50 sec.

that was at least not changed for several hours. The large pressor impulses remained apparently unchanged. If one injected rapidly a quantity which was two or three times larger than that given above, one obtained in the majority of cases also a disappearance or considerable reduction of the pressor impulses. These, however, returned successively in the course of five to ten minutes, while the chemical impulses had definitively disappeared. On rapid intrasinusal injection of 0.5—0.75 ml of this acid a lasting disappearance of both the chemical impulses and the pressor

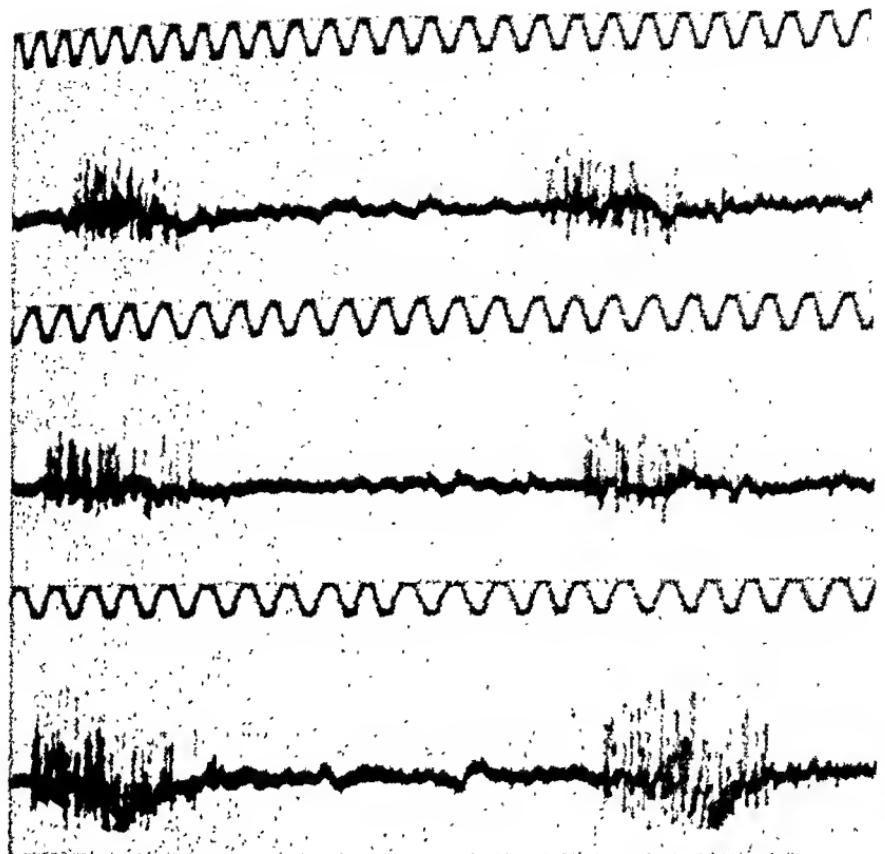
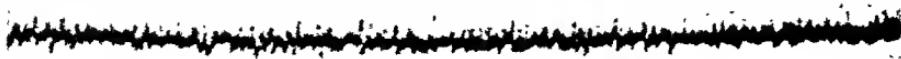


Fig. 17 b. Same experiment as in fig. 17 a but after intrasinusal injection of 0.10 ml 0.5 N acetic acid. D. Slight over-ventilation with air, BP 110 mm Hg. E. Same ventilation but with 10.5 % oxygen in nitrogen, BP 115 mm Hg. F. After intravenous injection of 1 mg lobeline hydrochloride, BP 150 mm Hg. Time: 1/50 sec.

impulses was obtained. As emerges from this, there is a rather big difference between the amount required to eliminate only the chemoreceptors and the amount that definitively destroys both chemo- and baroreceptors.

Fig. 17b shows the effect of 0.10 ml of acid intrasinusally. In comparison with the control (Fig. 17a) one sees here how the hypoxemia no longer provokes the ordinary increase of the chemical impulses. This is also the case after intravenous injection of 1 mg of lobeline hydrochloride, which before the elimination

A



B



C

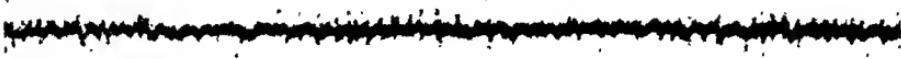


Fig. 18. Cat 2.9 kg. Artificial respiration air. Recording of the action potentials from the right depressor nerve. A. Control. BP 90 mm Hg. B. After injection via a catheter with the tip inside the aortic valves of 0.10 mg lobeline hydrochloride. BP 110 mm Hg. Thereafter elimination of the chemoreceptors with 0.30 ml 0.5 N acetic acid. C. Same injection of lobeline hydrochloride as in B. BP 105 mm Hg.

of the chemoreceptors caused the ordinary increase of the chemical impulses lasting for several minutes. Now one obtains only a considerable increase of the pressor impulses, which is connected with the increased arterial blood pressure. There can thus in this case not have been any paralysing effect on the nerve itself, but only the chemoreceptors as well as the eventual synapse formation (according to EULER, LILJESTRAND and ZOTTERMAN) have been selectively eliminated.

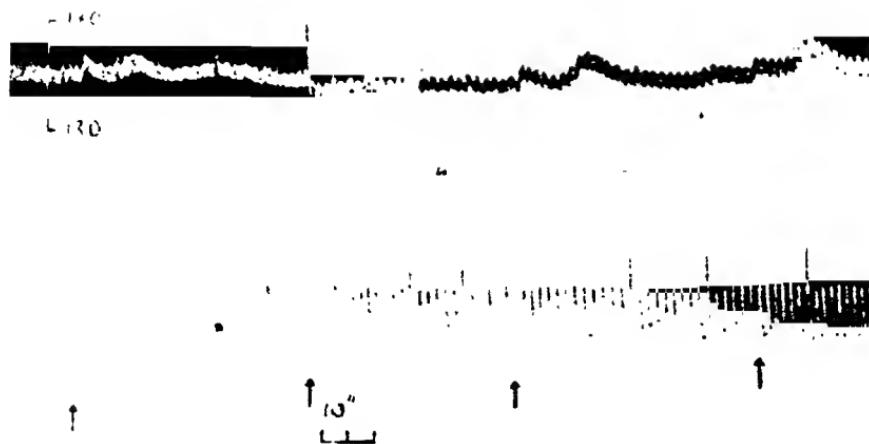


Fig. 19. Cat 2.1 kg. After bilateral sinus denervation. Upper curve blood pressure, lower curve respiration recorded with a pneumograph. The first arrow indicates intracardial injection of 0.3 mg lobeline hydrochloride. At the second arrow the chemoreceptors of the aorta are eliminated with 0.30 ml acid. The third and fourth arrows indicate the point of time for intracardial injection of 0.3 and 0.5 mg respectively of lobeline hydrochloride.

### 3. Aortic body.

Analogous conditions, as was expected, could be demonstrated from the aortic body on intracardial injection through the catheter, whose tip was placed inside the aortic valves. Fig. 18 shows the effect of 0.1 mg of lobeline hydrochloride before (B) and after (C) the injection of 0.30 ml of acid. Nor was this effect of the acid reversible for several hours, i. e. for the period during which the effect was checked. Fig. 19 shows, in an animal with denervated sinuses, the effect on the respiration and blood pressure of an intracardial injection of lobeline before and after the elimination of the chemoreceptors of the aorta.

With this method one thus has a possibility of selectively eliminating the chemical, reflexogenic excitability while the pressor regulation is unaffected.

## VII. The distribution of the effect of specific stimuli between the carotid and aortic bodies

As all earlier experiments have shown, the reflexogenic stimulus from the chemoreceptors in the carotid bodies excreises the main effect, while those from the aortic body are of more subordinate importance.

In his investigation, COMROE (1939) gives a scheme of this percentual distribution between the carotid bodies and the aortic body in the dog of the effect of hypoxemia on the respiration and blood pressure in connection with denervation experiments. He finds that the carotid bodies alone are able to produce the same stimulation upon the respiration as when the animal is intact, while when only the aortic body is functioning one only obtains an increase of 25 % in the respiration, whereas the rise in the blood pressure is now more clearly marked than in the intact animal, owing to the fact that the regulators of the blood pressure from the sinuses have been eliminated by the denervation. In estimating the distribution of the effect of hypoxemia on the blood pressure and respiration between the chemosensitive zones in the intact animal he finds that about 15 % of the rise in the blood pressure is due to the reflexes from the sinuses, while about 85 % comes from the reflexes of the aorta region. The effect on the respiration is so distributed that 90 % is localized to the carotid bodies, while only 10 % goes over the aortic body.

### 1. Technique and Procedure:

In the investigation of this distribution of the effect the chemoreceptors have as usual been eliminated with 0.5 N acetic acid.

The experiments were performed, as before, on cats, which

were first narcotized with ether and afterwards received 0.05—0.07 g of chloralose per kilogram of body-weight. A tracheal cannula was introduced in the usual way. The blood pressure was registered from the femoral artery with an Hg-manometer. Cannulae were introduced into the lingual arteries and a catheter was passed through the common carotid artery to the heart in the way already described.

The respiration was recorded according to the principle described by HALDANE and PRIESTLY (1905) with a method described by EULER and LILJESTRAND (1936), the animal being placed in an air-tight box which was connected with a spirometer. The tracheal cannula was connected with a glass tube which left the box through an air-tight opening in one wall and was connected with a Müller valve with slight resistance, some millimeters of water. The ventilation was obtained as a product of the spirometer reading and the respiratory frequency. The arterial cannula passed through a hole in the wall, enabling simultaneous registration of the blood pressure.

## 2. Hypoxemia.

When the animals experimented upon were allowed for 2 minutes to breathe spontaneously a gas mixture poor in oxygen, 7.3 % oxygen in nitrogen, before and after the elimination in turn of the chemoreceptors in the aorta region and in the sinuses, the following results were obtained in nine typical experiments.  
a) *The respiration.* The stimulating effect on the respiration of the intact animal amounted to about 54 % as compared with the ventilation with air. If the chemoreceptors in the aortic body were eliminated one still obtained a rise in the respiration of 43 % on respiration of the gas mixture poor in oxygen. If after this one destroyed also the chemoreceptors in the sinuses, the usual reduction and standstill of the respiration appeared (Fig. 20) that is obtained with hypoxemia after severance of the sinus nerves and the vago-depressors. If air is not then quickly supplied the cat dies.

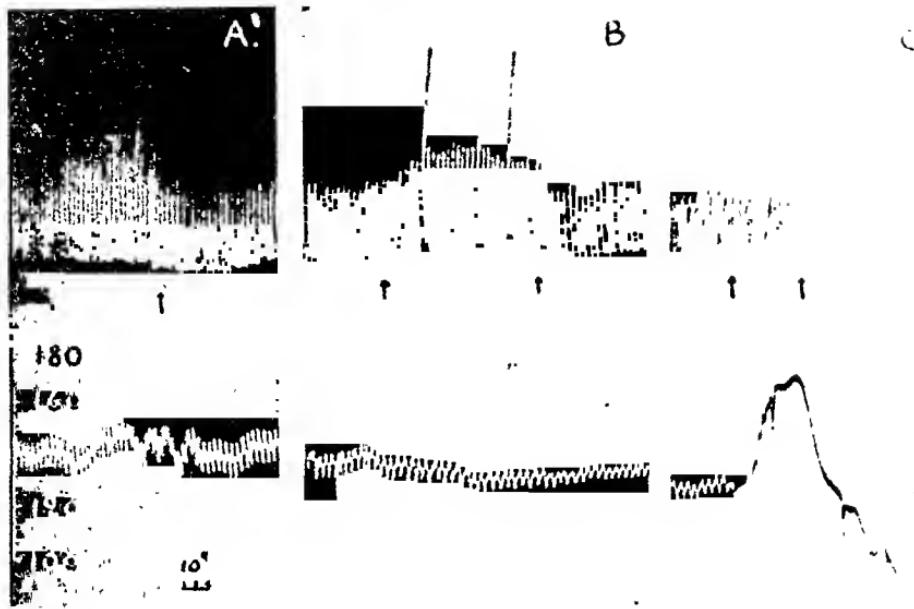


Fig. 20. Cat 3.1 kg. Upper curve recording of respiration by means of a body plethysmograph, lower curve blood pressure. A. Spontaneous respiration of 7.3 % oxygen in nitrogen. B. After elimination of the chemoreceptors in the aortic body. Spontaneous respiration of 7.3 % oxygen in nitrogen. C. After elimination also of the chemoreceptors in the carotid bodies. Spontaneous respiration of 7.3 % oxygen in nitrogen.

If the chemoreceptors in the sinuses were destroyed first one obtained no increase in the respiration with hypoxemia that could be ascribed to the aortic body, but the ordinary reduction and standstill of the respiration appeared. From this it emerges that the chemoreceptors of the aorta alone cannot protect the cat in case of hypoxemia.

b) *The blood pressure.* If we now consider the blood pressure reactions during these experiments we find that the slight rise in blood pressure that appears in the intact cat on hypoxemia does not occur or is changed to a slight fall after elimination of the chemoreceptors of the aorta. If both these and the chemoreceptors of the carotid bodies are destroyed, hypoxemia causes, in the majority of cases, an initial rise in the blood pressure,

which occurs already before the respiration has been changed, but which is thereafter followed by a marked fall in the blood pressure (Fig. 20). This initial rise must, to begin with, be considered to be due to a direct effect of the poorly oxygenated blood on the vaso-motor centres. But the rise in blood pressure also remains for a while after the respiration has diminished or stopped, so that one must also consider an effect of an increase of carbon dioxide on the centres. The marked subsequent fall in the blood pressure is a sign that these centres and the heart are failing.

SELLADURAI and WRIGHT (1932 a) also observed in some cases this initial rise in blood pressure before the appearance of the definitive fall in blood pressure. In other cases they obtained only the fall in blood pressure, without any preceding rise, in experiments on cats. This has also been observed in the investigations referred to above. EULER and LILJESTRAND (1936), in experiments on cats, obtained only the fall in blood pressure, whereas on the other hand, they found in dogs the initial rise before the respiration had changed, but followed thereafter by the usual fall in blood pressure. They therefore assumed that the vaso-motor centre in cats was especially sensitive to hypoxemia and that it accordingly soon failed.

### 3. Lobeline.

a) *The respiration.* The quantitative distribution of the effect of lobeline on the respiration has been studied before and after the destruction of the chemoreceptors. Lobeline hydrochloride has been given intravenously in an amount of 0.0 mg, which gave rise to a suitable marked increase of the respiration. This increase of the ventilation amounted in the intact animal in nine typical experiments to 41 % as compared with the original value. After elimination of the receptors in the aortic body the stimulating effect of lobeline on the respiration diminished to 30 %. If after this the chemoreceptors in the sinuses were destroyed, no effect on the respiration was obtained (Fig. 21). If the chemo-

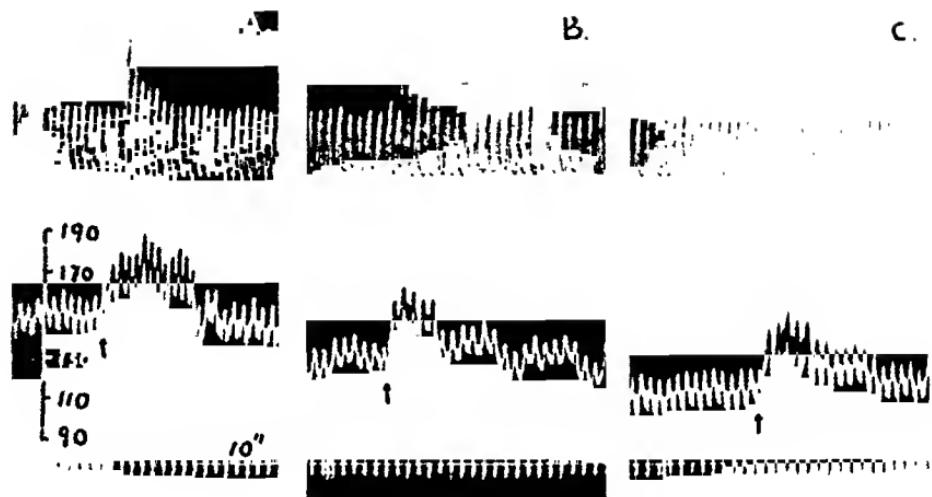


Fig. 21. Cat 3.3 kg. Upper curve recording of respiration with a body plethysmograph, lower curve blood pressure. At the arrows intravenous injection of 0.9 mg lobeline hydrochloride. A. The effect on the intact animal. B. After elimination of the chemoreceptors of the aorta. C. After elimination of the chemoreceptors in the sinuses.

receptors were eliminated in the reverse order the result was the same.

b) *The blood pressure.* Apart from the stimulating effect on the respiration, lobeline also has such effect on the blood pressure. This effect has been mainly interpreted as central, although a part of it is undoubtedly to be considered as caused by a stimulus from chemoreceptors in the carotid bodies (HEYMANS, BOUCKAERT, EULER and DAUTREBANDE 1932, EULER, LILJESTRAND and ZOTTERMAN 1939) and in the aortic body (COMROE 1939). Ligation of the suprarenal bodies did not prevent the rise in blood pressure.

If one compares the rise in blood pressure caused by lobeline in the intact animal with the rise that is obtained after the receptors in the glomus aorticum have been destroyed, one gets, in the majority of cases, a slight reduction. Subsequent elimination of the chemoreceptors in the sinuses caused in most cases a further reduction in the rise in blood pressure, but the results are so varying that one cannot give any exact percentual reduction.

#### 4. Hypercapnia.

In this connection the effect of carbon dioxide on the respiration and blood pressure before and after the selective elimination of the chemoreceptors was also investigated.

- a) *The respiration.* In order to ascertain to what extent the eventual respiration-stimulating effect of carbon dioxide was conditioned by stimulation of the chemoreceptors, the animals were allowed to breathe spontaneously a gas mixture of 7.3 % of carbon dioxide in air before and after the chemoreceptors had been eliminated. If the aortic body was destroyed one obtained a reduction that was so insignificant that it could not be estimated with any certainty. On the other hand, one obtained a reduction of about 16 % if the receptors in the carotid bodies were destroyed. Bearing in mind that also the alveolar carbon dioxide tension had probably increased, and thus caused a stronger central effect after elimination of the chemoreceptors (EULER and LILJESTRAND 1936), one is probably well advised to consider this figure as possibly somewhat too low.
- b) *The blood pressure.* On inhalation of the carbon dioxide-rich gas mixture the blood pressure was either unchanged or showed an extremely slight rise. No certain change after destruction of the chemoreceptors was obtained.

## VIII. The reflex effect on the respiration of intrasinusal pressure changes

It has long been known that a compression of the common carotid arteries causes an increase in the respiration. To begin with, this was considered to be due to an impaired central blood supply. The discovery of the aortic and sinus reflexes led to a closer investigation of this phenomenon. It was then found that a rise in pressure in the sinus region caused an inhibiting effect on the respiration, while a reduction of the pressure in the same region, e. g. by compressing the carotids, gave rise to an increase in the respiration (HEYMANS and BOUCKAERT 1930, KOCH and MARK 1931, GOLLWITZER-MEIER and SCHULTE 1931, SCHMIDT 1932, HEYMANS, BOUCKAERT and DAUTREBANDE 1932). GEMMILL, OVERSTREET and HELLMAN (1933), however, did not find any change in the respiration in non-anaesthetized dogs on compression of the carotids, either before or after sinus denervation. HEYMANS and HEYMANS (1927) were the first to show the inhibiting effect on the respiration of the rise in pressure in the reflexogenic zone of the aorta.

HEYMANS and BOUCKAERT asserted that the respiratory centre was subject to a reflexogenic influence from the aorta and sinus regions, and they were able to show, in perfusion experiments on the sinus, that the respiration could be reduced and even stopped if the perfusion pressure was raised sufficiently, whereas a low perfusion pressure increased the respiration. These effects did not appear after bilateral sinus denervation. Nor was it possible to obtain the usual increase in the respiration after compression of the common carotid arteries if the sinus nerves were severed. They considered that this effect on the respiration was produced from the baroreceptors. SCHMIDT (1932) assumed that the effect on the respiration of blood pressure changes in

the sinus region was presumably due to other factors than those which affect the circulation, as the respective intensities of the two phenomena were often completely independent of each other. This assumption has received support in several investigations. EULER and LILJESTRAND (1936, 1937) considered that the changes of the circulation in this region caused by variations in the blood pressure changed the chemical effect of the blood. They came to the conclusion, therefore, that the reflexogenic effect on the respiration in connection with various intrasinusal pressures was not elicited from the baroceptors but from the chemoreceptors, owing to a change in the chemical effect of the blood. Thus, low intrasinusal pressure leads to hypoxemia and hyperpnoea, high intrasinusal pressure gives rise to improved circulation and a disappearance of the hypoxic and carbon dioxide stimuli, which quickly leads to a reduction of the ventilation and frequently to apnoea until the carbon dioxide losses have been made good. EULER, LILJESTRAND and ZOTTERMAN (1939), recording the action potentials from the sinus nerve of the cat, were able to show a considerable increase of the chemical impulses on compressing the carotids; but this increase of impulses disappeared if they over-ventilated the animal with oxygen, and must therefore be considered to be due to stimulation of the chemoreceptors. RUDBERG (1938) showed that the stimulating effect on the respiration following compression of the carotids was increased if the blood pressure was reduced below a critical value. The same author (1940) showed that this could be counteracted to a certain extent by inhalation of oxygen. Further investigations by EULER and LILJESTRAND (1940) showed that on perfusion of the sinus region with blood poor in oxygen (saturation = 33 %), in contradistinction to blood rich in oxygen, a reduction of the intrasinusal pressure had no effect on the respiration, as in this case the chemoreceptors, presumably already at high perfusion pressure, were maximally stimulated by the hypoxemia.

WINDER (1938), by embolization of the carotid body with a lycopodium suspension, attempted to eliminate the chemoreceptors, but respiratory reflexes still appeared, however, on intrasinusal pressure variations. MARRI and HAUSS (1939) carried

out an investigation on the effect of certain factors on the respiratory reflexes in connection with intrasinusal pressure variations employing a method which was previously used by HEYMANS, DONATELLI and SHEN (1938). A rubber bag or a bag made out of a vein was introduced into the sinus, and they considered that by this means, by varying the pressure in the bag, they could release pressor reflexes without affecting the chemoreceptors. By increasing or reducing the pressure they were able to affect the respiration. This work, as well as a later one by GRIMSON and SHEN (1939), in which the same method was employed, has been exposed to a certain criticism, as with this procedure the blood supply to the carotid body must have been affected.

BJURSTEDT and HESSER (1942) used a special cannula, thanks to which the sinus regions could be relatively well supplied with oxygenated blood even at a low perfusion pressure. In this connection they were able to show that endosinusal pressure changes did not necessarily cause any change in the respiration even if typical blood pressure reflexes appeared. Under certain circumstances the change in pressure could result in a variation in the respiration, even if no blood pressure reflexes appeared. They therefore considered that the respiratory changes that arise in connection with intrasinusal pressure changes are released from the chemoreceptors. BJURSTEDT and EULER (1942) also found that under normal conditions the respiration was not affected reflexogenically from the baroceptors in the sinus and aorta regions, although they were able definitely to confirm the occurrence of such an influence after severance of the vago-depressors.

To the method of selectively eliminating the chemoreceptors with acid it might be objected that also the baroceptors are damaged. That this is scarcely the case, however, has already been pointed out in connection with recording of the action potentials from the sinus and depressor nerves. Further support for the assumption that they are intact is given in the following experiments with simultaneous recording of the respiration and blood pressure, in which, as a test of the intactness of the pressor-reflexes after elimination of the chemoreceptors, the effect on

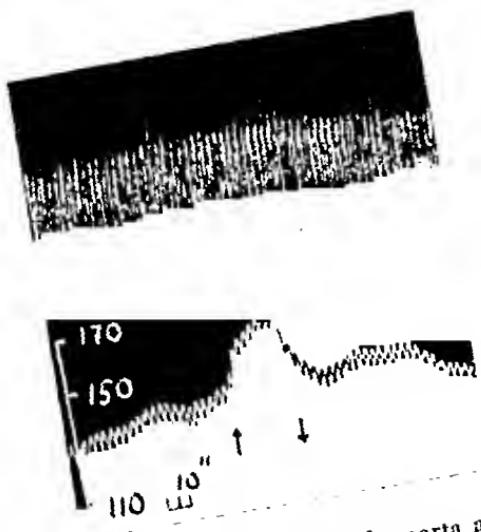


Fig. 22. Cat 3.1 kg. The chemoreceptors in the aorta and sinuses selectively eliminated with 0.5 N acetic acid. For 2 minutes the cat was allowed to breathe pure oxygen, after which the left common carotid was compressed. Right common carotid previously closed by the catheter.

the blood pressure has been controlled on compression of the carotid artery. It was possible to regulate this compression outside on the blood pressure through the compression, the animals were first allowed to breathe pure oxygen for about 2 minutes (EULER, LILJESTRAND 1943). If a clear rise in blood pressure was then obtained on compression one could be sure that the pressor reflexes were intact (Fig. 22). As further support for the assumption that the reflexogenic increase in respiration on a change of the intrasinusal pressure, but with intact vagi is not caused by the change in pressure, but is entirely due to the chemoreceptors, it proved that one did not obtain any change in the respiration on compression of the carotid artery when the chemoreceptors were selectively eliminated.

## IX. Adrenaline apnoea

If one injects a sufficiently large amount of adrenaline intravenously, it proves that the ordinary rise in blood pressure is followed by a reduction of the respiration, which is sometimes so pronounced that apnoea occurs. This phenomenon was first described by OLIVER and SCHÄFER (1895), who injected extract of the supra-renal body into dog and rabbit. A similar effect on the respiration was also obtainable if one caused a quick rise in blood pressure by compressing aorta abdominalis (ROBERTS 1921). He explained that the adrenaline effect on the respiration was caused by a direct effect on the medulla through a constriction of the vessels in this region. This led to a paralysis of the centre through hypoxemia or accumulation of metabolites. MELLANBY and HUGCERT (1923) also arrived at this view. This explanation, however, is not easily reconciled with the apnoea which can be provoked on compression of the aorta. Since the sinus mechanism has been discovered new ways of explaining the adrenaline apnoea have been sought. HEYMANS and BOUCKAERT (1930) and WRIGHT (1930) showed that it is mainly reflexogenically conditioned, for after denervation of the carotid sinuses and severance of the vagi adrenaline caused practically no, or an extremely slight, effect on the respiration. HEYMANS and BOUCKAERT interpreted the phenomenon to mean that the adrenaline apnoea is due to a reflexogenic inhibition of the respiratory centre provoked by the rise in blood pressure in the aorta and sinus regions; thus a stimulation of the baroceptors. The earlier cited literature on the reflex effect of intrasinusal pressure changes on the respiration tends rather to support an explanation of the adrenaline apnoea as being due to the increased

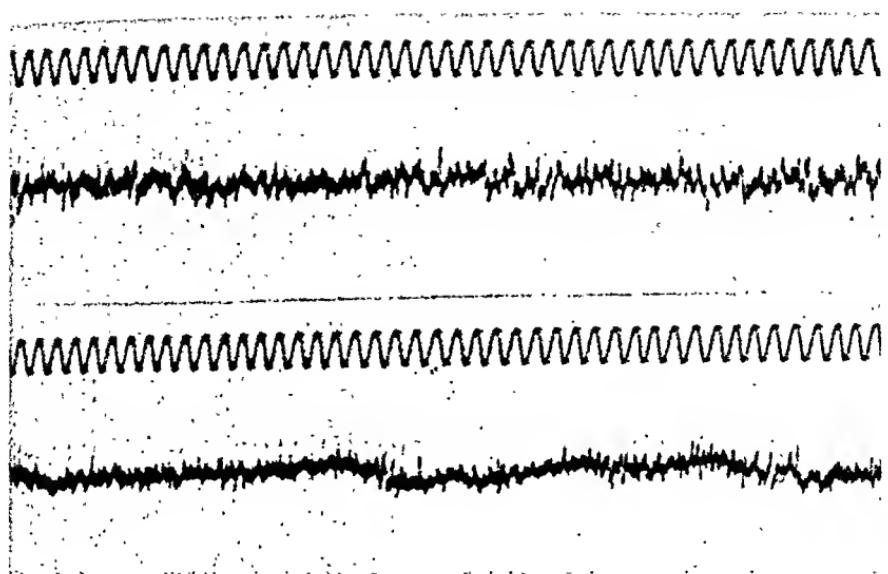


Fig. 23. Cat 3.2 kg. Recording of the action potentials from the right depressor nerve. Artificial respiration air. A. Control, BP 140 mm Hg. B. After intravenous injection of 15 µg adrenaline, BP 200 mm Hg.

arterial blood pressure, which causes a better circulation through the carotid and aortic bodies and thus a reduction of the effect of the chemoreceptors in these regions. GERNANDT, LILJESTRAND and ZOTTERMAN (1945) gave further evidence that the adrenaline apnoea was caused by a reduction in the activity of the chemoreceptors. In experiments on cats they showed that the apnoea could be prolonged if the animal was allowed to breathe oxygen, and more or less suppressed if instead it was allowed to breathe oxygen-poor or carbon dioxide-rich gas mixtures. Records of the action potentials from Hering's nerve showed that the chemical impulses were strongly reduced during the adrenaline apnoea. Taking these last investigations as our point of departure, it thus appears rather doubtful that the adrenaline apnoea should be caused by a directly inhibiting effect from the baroceptors in the reflexogenic sinus and aorta regions.

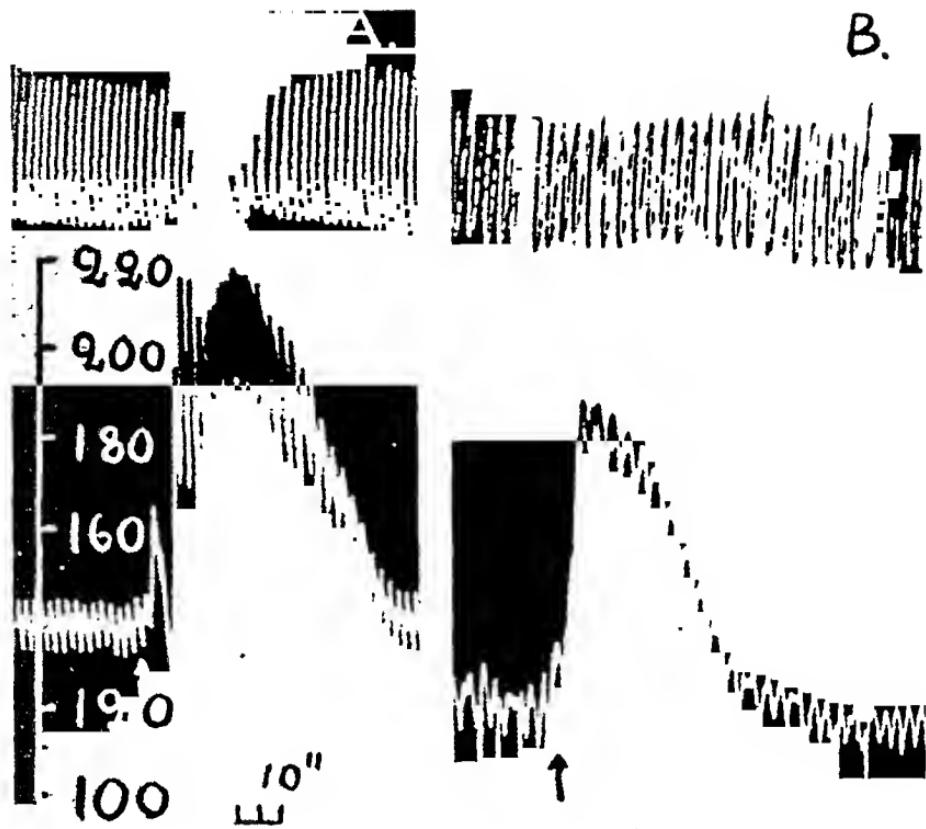


Fig. 24. Cat 3.1 kg. Same experiment as in fig. 22. Upper curve recording of respiration with a body plethysmograph, lower curve blood pressure. The arrows indicate intravenous injection of 20 µg adrenaline. Between A and B the chemoreceptors in the aorta and sinuses are eliminated.

### 1. The effect of intravenous injection of adrenaline on the activity in the chemoreceptive fibres in the depressor nerve.

On recording the action potentials from the depressor nerve, the same considerable reduction of the small impulses after intravenous injection of adrenaline (Fig. 23) was obtained. As has already been pointed out, it is technically impossible to destroy with mechanical means the pressoreceptive fibres without

at the same time injuring the chemoceptive fibres. The pressor impulses thus remain, and one sees how they increase quite naturally after the injection of adrenaline.

## 2. The effect on the respiration of intravenous adrenaline injection after elimination of the chemoreceptors.

According to the previously described method, the sensitivity of the chemoreceptors was extinguished with acid, after which it was attempted to ascertain whether adrenaline can then cause any apnoea. It proved that after the above procedure one could not obtain any adrenaline apnoea on intravenous injection of 20 µg of adrenaline, which otherwise caused a clear apnoea (Fig. 24). Compression of the carotid artery after spontaneous breathing of pure oxygen for 2 minutes gave rise to the usual rise in blood pressure. Sometimes, however, one still obtained a slight remaining adrenaline effect on the respiration after the destruction of the chemoreceptors. HEYMANS and BOUCKAERT, WRIGHT and GERNANDT, LILJESTRAND and ZOTTERMAN also found this effect after severance of the sinus nerves and the vago-depressors. The last-mentioned authors also observed that this slight adrenaline effect on the respiration remained during the inhalation of oxygen. It would seem most natural to assume that it is caused by a central effect, although the actual mechanism is unknown.

These results give further support for the view that the adrenaline apnoea is caused by a reduction or disappearance of the reflexogenic, chemical stimulation of the respiration, and not by any inhibiting effect exerted on the centre from the baroceptors.

## X. Tonic chemoreflex respiratory stimulation

A reduced respiration after the elimination of the sinus mechanism is a phenomenon that has been observed by several authors in experiments on animals. SELLADURAI and WRIGHT (1932 b), in experiments on cats narcotized with chloralose found that sinus denervation caused a reduction of the ventilation amounting as an average to 22 %. In experiments on decerebrated cats they obtained on denervation a reduction of 33 %, doubtless partly owing to the fact that the carotids were ligated and the respiration was in consequence reflexly increased before the denervation. STELLA (1936) found in anaesthetized dogs a similarly reduced respiration on functional elimination of Hering's nerves. Also EULER and LILJESTRAND (1936, 1940) observed this reduction in the ventilation and an increase in the alveolar carbon dioxide tension on severing the sinus nerves and the vago-depressors in experiments on dogs and cats. The effect was as a rule reduced but not eliminated if the animals were allowed to breathe pure oxygen. GESELL, LAPIDEZ and LEVINE (1940) and SCHMIDT, COMROE and DRIPPS (1939) also found this reduction in the respiration after elimination of the sinus mechanism. These experiments thus show that under the conditions obtained there is a flow of impulses in the sinus nerves which affects the ventilation. This flow of impulses is elicited by hypoxemia and carbon dioxide according to the records of the action potentials from the sinus nerve.

To obtain a quantitative idea of the magnitude of this tonic stimulation from the chemoreceptors on the respiration in these experiments is very difficult, since severance of the sinus nerve breaks the afferent channel for the chemoreceptors as well as for the baroreceptors: and, as has been mentioned above, several authors

consider that from the baroreceptors there is produced a tonic, reflexogenic inhibiting effect on the respiration in agreement with the inhibition exerted on the blood pressure (HEYMANS and BOUCKAERT 1930, HEYMANS, BOUCKAERT and DAUTREBANDE 1930, KOCH and MARK 1931). These writers found that after bilateral sinus denervation and after severing both vago-depressors an increased respiration was obtained. Elimination of the tonic inhibition of the respiration would thus have a directly opposite effect on the absence of the tonic influence from the chemoreceptors. The reason for this difference of opinion is perhaps to seek in the change in the respiration that arises when the respiration-regulating function of vagus is eliminated. That the chemical factor is in any case far more important has been shown by BERNTAHL and WEEKS (1939), who cooled the chemoreceptors in connection with isolated circulation of these with blood at different temperatures. In this way they were able to obtain a selective elimination of these while the pressor reflexes were intact; and in experiments on dogs they found that an elimination of the tonic, reflexogenically stimulating effect from the chemoreceptors entailed a reduction of the ventilation amounting to 34 %.

The method of selectively eliminating the chemoreceptors in both the sinus and aorta regions with acid offers fresh possibilities of studying the tonic, reflexogenically stimulating effect on the respiration and blood pressure released from all the chemoreceptors that have any physiological importance. The pressor-reflexes can remain intact and function as before.

The animal was placed in the above-described body plethysmograph. The registration of the respiration was begun about an hour after the animal had been narcotized with ether and chloralose. It was therefore not necessary to reckon with any ether effect on the respiration. As GERNANDT (1943) pointed out also in experiments on cats, with this method of recording one obtains during the first hour a continuous successive reduction of the respiration, after which it remains relatively constant. In view of this fact the ventilation has not been estimated during this first hour.

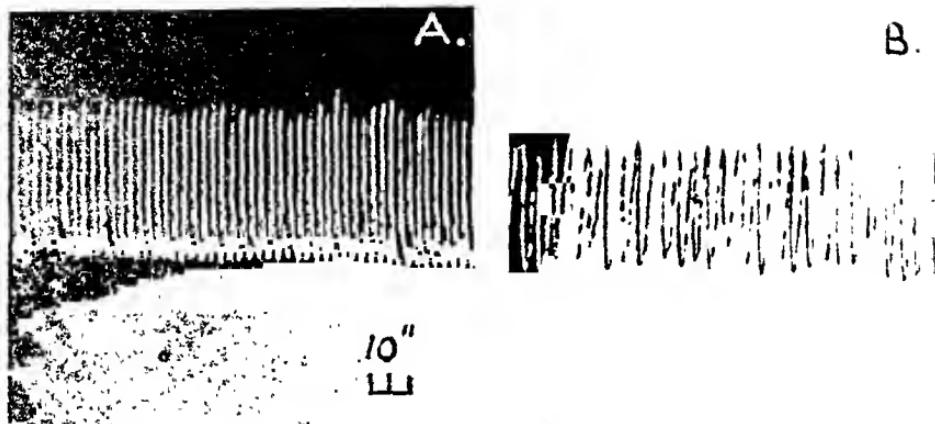


Fig. 25. Cat 3.1 kg. Registration of the respiration by means of a body plethysmograph. A. Spontaneous respiration of air. B. After selective elimination of the chemoreceptors in aorta and sinuses.

Before and after elimination of the chemoreceptors the effect of inhalation of 7.3 % oxygen in nitrogen was tested.

### 1. The effect on the respiration.

The magnitude of the ventilation was estimated in periods of 1 minute. The mean value in seven typical experiments on cats weighing between 2.5—3.4 kg was a ventilation of  $0.56 \pm 0.03$  liters per minute. The respiratory frequency was  $11.9 \pm 0.76$  breaths per minute.

If after this the chemoreceptors in the aorta region were eliminated, the ventilation was reduced to  $0.49 \pm 0.02$  liter per minute, with a respiratory frequency of  $10.6 \pm 0.64$  breaths per minute. A selective elimination of the chemoreceptors in the sinuses caused a further reduction of the respiration to a value of  $0.36 \pm 0.02$  liters per minute. The respiratory frequency was now  $8.6 \pm 0.62$  breaths per minute (Fig. 25). This thus shows that the ventilation is reduced by about 36 % if the reflexogenically stimulating effect from the chemoreceptors under these conditions is eliminated. If one considers the distribution of this effect between the chemoreceptors in the aortic body and the carotid bodies respectively

one arrives at the result that out of this 36 %, 13 is conditioned over the former while 23 is produced by the latter.

In view of the fact that the circulation in the right common carotid artery was cut off by the catheter and the sinus on this side did not obtain blood through the ordinary channels, elimination of the chemoreceptors was also effected in the reverse order. The chemoreceptors in the carotid bodies were first eliminated, and after this the catheter was introduced into the common carotid artery and acid was injected to destroy the chemoreceptors of the aorta. It was not possible, however, to observe any difference in the results obtained. Nor was any definite change in the results given above obtained after severing the right sinus nerve with subsequent elimination of the functioning chemoreceptors. This is in agreement with the observation that denervation of the one sinus scarcely causes any change, as the other sinus exercises a compensatorily increased function.

## 2. The effect on the blood pressure.

The elimination of the chemoreceptors at the moment of injection caused a very brief rise in blood pressure, which thereafter returned to the original level. This rise in the blood pressure is probably to be considered as mainly due to a general sensitizing stimulating effect of the acid, as one sees with what strong jerks the animal reacts. The disappearance of the pressor impulses for some moments that is sometimes observed when recording the action potentials may also be a contributing cause through the temporary absence of the pressure regulation. Theoretically, the elimination should entail a lowering of the blood pressure through the removal of the tonic stimulation of the chemoreceptors with effect on the blood pressure. But apart from this temporary rise in blood pressure the pressure remained constant, if one excepts the usual gradual lowering that is obtained in experiments that are continued for several hours. This is of course also to be expected, as the pressor regulation is intact. The reduction in the ventilation due to elimination of



Fig. 26. Cat 3.0 kg. Upper curve recording of respiration with a body plethysmograph, lower curve blood pressure. A. Between the arrows clamping of the carotid artery after 2 minutes' spontaneous respiration of pure oxygen. B. The chemoreceptors in the sinus and aorta regions selectively eliminated with acid. Between the arrows clamping of the carotid artery after 2 minutes' spontaneous respiration of pure oxygen. C. After further injection of acid elimination also of the baroreceptors. Between the arrows clamping of the carotid artery after 2 minutes' spontaneous respiration of pure oxygen.

the chemoreceptors is also followed, as EULER and LILJESTRAND (1936) have shown in connection with denervation experiments, by an increase in the alveolar carbon dioxide tension. This might possibly be able to cause a rise in the blood pressure; but one must, however, also bear in mind that a certain hypoxemia may arise, which in animals with eliminated chemoreceptors causes a lowering of the blood pressure. It is possible that these two factors compensate each other, so that the blood pressure remains constant.

In this connection it also proved suitable to return to the question of the importance of the baroreceptors for the regulation of the respiration. At first the chemoreceptors were destroyed with acid, and the usual reduction of the ventilation appeared, after which 0.5 ml acid was quickly injected into each sinus as well as 0.75 ml via the catheter. This immediately caused a strong,

permanent rise in the blood pressure, which showed that the depressor reflexes were eliminated. A further possibility of ascertaining whether the depressor reflexes were eliminated lay in a compression of the carotid artery. Fig. 26 shows the effect on the blood pressure and respiration of this compression, after the animal had first been allowed to breathe 100 % oxygen spontaneously for 2 minutes in order to exclude any central effect. Fig. 26 A shows the effect of the compression in the intact animal, B after the chemoreceptors have been destroyed, in C also the baroceptors have been destroyed, and then, as usual, one does not get this typical rise in blood pressure in connection with the clamping of the artery. It was not possible to obtain any increase in the ventilation indicating the elimination of any tonic inhibition exerted from the baroceptors. The ventilation in the intact animal before the compression was 0.42 liters per minute, with a respiratory frequency of 8.8 breaths per minute. After destruction of the chemoreceptors the ventilation was as usual reduced, and amounted then to 0.34 liters per minute, with a respiratory frequency of 7.8 breaths per minute. This ventilation was *not* changed after the elimination of the baroceptors. These results thus constitute direct evidence against the assumption that the baroceptors exercise any tonic inhibiting effect on the respiration of the cat.

## XI. Summary

Taking previous investigations on the existence of a chemosensitive region in the tract around aorta ascendens, this region has been made the object of closer studies.

In an anatomical survey a more detailed account has been given of the localization of a number of cell groups of paranglionie type around the aorta and the pulmonary artery. These so-called paraganglia are in part sympathetic, consisting chiefly of chromaffin cells, and in part parasympathetic, which have the same morphological structure as the formations occurring in the sinus region.

The chemo-sensitive zone of the aorta in the cat has been localized to the region below the aortic arch in the connective tissue between the aorta and the pulmonary artery. By mechanically destroying this formation as well as the afferent nerve fibres proceeding therefrom, one obtained, on recording the action potentials in the depressor nerve, the afferent nerve from the aortic body, a complete elimination of all chemical impulses, while impulses from the baroreceptors still continued to come. Owing to the anatomical conditions it was not possible in this way to destroy only the afferent fibres of the baroreceptors, without the afferent fibres of the chemoreceptors being at the same time more or less involved in the process.

By the local application of certain specific stimuli it was possible to confirm that the aortic body in the cat obtains its blood supply through the coronary vessels. This local application also enabled one to obviate the possibility of other paraganglia around the aorta reflexly affecting the respiration.

Action potentials in the depressor nerve released from the chemoreceptors in the aortic body under the influence of hypoxemia, carbon dioxide and specifically stimulating substances (lobeline, piperidine etc.) have been recorded. These potentials

are of the same nature as those one obtains under similar conditions on recording from the sinus nerve.

A method of eliminating the chemoreceptors in the aortic body and the carotid bodies by injecting locally a small amount of 0.5 N acetic acid is described. In this connection the chemoreceptors are destroyed, while the baroceptors remain intact. This fact was checked partly by recording the action potentials from the afferent nerves and partly also by controlling the blood-pressure reflexes on clamping the carotid artery. Larger amounts of acid were also able to destroy the baroceptors.

After elimination of the chemoreceptors no effect of carotid compression on the respiration was obtained. No sign that the intact baroceptors in the cat can have any effect on the respiration has been observed. As further support for this it was possible to show that the ventilation did not undergo any change when the baroceptors were destroyed in an animal with chemoreceptors already eliminated.

A selective elimination of the chemoreceptors caused a reduction in the ventilation by about 36 %. The chemoreceptors thus exercise a tonic, reflexogenic stimulation of the respiration. It was possible to measure the relative effects on respiration and blood pressure which were exerted by hypoxemia and carbon dioxide and such a specific stimulus as lobeline. This was done after first eliminating the chemoreceptors of the aortic region and then also eliminating those in the carotid sinus regions. This separation of aortic and carotid sinus effects demonstrated the subordinate importance of the aortic body as compared with the carotid bodies.

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